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ORIGINAL ARTICLE

Novel UPLC-MS/MS method for standardization of niazimicin content in edible seeds and leaves of *Moringa oleifera* Lam

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Abstract

Moringa oleifera* (*MO*) is a highly nutritious plant, whose leaves and seed pods are consumed in Africa, Asia, sub-Himalayan regions and South America. A novel ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for the standardization of the bioactive thiocarbamate compound from *MO*, niazimicin (NZ) in seeds and leaves, is developed, optimized and validated according to the International Conference on Harmonization (ICH) guidelines, using desipramine as the internal standard. Multiple reaction monitoring detection of transitions 358.05 > 106.86 and 266.38 > 193.04 with collision energy of 25V and 40V, respectively were used. A gradient was optimized at 35-55%B in 7 min, where mobile phase A is aqueous 0.1% formic acid and B is 0.1% formic acid in acetonitrile. The method proved to be linear in the range of 0.05-100 μg/mL, precise, robust and accurate, with LOD and LOQ of 0.02 and 0.05 μg/mL, respectively. *MO* seeds were found to contain double NZ content (620 mg% ±3.2%) compared to leaves, and the methylene chloride fraction of seeds comprised triple the amount in ethyl acetate fraction (450 mg% ±2.4%). Results emphasize that seeds of *MO* are a much richer source for NZ than the most commonly edible and marketed leaves extracts. The reported method can be used for standardization and quality control of the seeds and leaves NZ content.

Keywords: Leaves, *Moringa oleifera*, Niazimicin, Seeds, UPLC-MS/MS

1. Introduction

Moringa oleifera Lam. (*MO*) is a popular member, widely geographically distributed, of the monogeneric family *Moringaceae*, which includes 13 different species. Almost all *Moringa* species are native to the sub-Himalayan regions (India, Pakistan, Bangladesh and Afghanistan), from where they have been introduced to many warm countries such as Egypt [1]. *MO* leaves, flowers, roots and fruits, have traditionally been used in diet where leaves are eaten as greens in salads in Malaysia and the Indian sub-continent. *MO* seed oil is highly edible and resembles olive oil in its fatty acid composition [2]. *MO* is known for its high antioxidant potential owing to its flavonoids and phenolic acids content [3], as well as possessing antimicrobial, hypotensive and anticancer effects due to its glucosinolates content [4].

Niazimicin (NZ), a thiocarbamate glycoside produced upon enzymatic hydrolysis of glucosinolates by myrosinase enzyme, is one of the mustard oil glycosides, which are very rare in nature. It has been previously reported to show several bioactivities. The compound was first identified by Faizi et al. as a spasmolytic (by inhibiting acetylcholine and histamine-induced ileal contractions in guinea pigs) and hypotensive agent having bradycardiac effects as seen both *in vivo* in Wistar rats and *in vitro* in guinea pigs atria and rabbits aorta (by inhibiting K+-induced contractions). NZ was also shown to inhibit

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the spontaneous contractions of the rat uterus, mediating its effects through a muscarinic receptor-independent mechanism [5-7]. In addition, NZ was proposed to be a potent chemo-preventive agent in chemical carcinogenesis by Guevara et al. [8]. Through *in vivo* testing, it was found to have potent antitumor promoting activity in the two-stage carcinogenesis in mouse skin using 7,12-dimethylbenz[a]anthracene (DMBA) as the initiator and 12-*O*-tetradecanoyl-phorbol-13-acetate.

(TPA) as the tumor promoter. On top of these activities, NZ has shown an anti-microbial activity against *Staphylococcus aureus* (*S. aureus*) upon testing it as well against *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Candida albicans* (*C. albicans*) and *Aspergillus niger* (*A. niger*), as reported by Jeon et al. [9].

Several HPLC- UV or MS methods [10–15] were developed and validated for qualitative and quantitative analysis of a variety of extracts or phytoconstituents from different parts of *MO*. The major analytes of interest previously investigated included niazirin & niaziridin, crypto-chlorogenic acid, isoquercetin & astragalin, α- and γ-tocopherol, quercetin, rutin & kaempferol, glucosinolates & phenolics (flavonoids, anthocyanins, proanthocyanidins & cinnamates). Although NZ has an important biological activity as described above, no chromatographic analysis method has ever been reported for its identification or quantitation in *MO*. In addition, no comparison has been ever undertaken between its levels in seeds and leaves, hence the nutritional value of seeds and leaves has not been contrasted. Since LC-MS/MS methods offer specific and selective qualitative and quantitative methods using lower amounts of sample compared to other techniques, in this study we report for the first time a fast and sensitive validated UPLC-MS/MS method for quantitation of NZ in seeds and leaves of *MO*. This can be very useful in future pharmaceutical applications such as quality control studies, as NZ tends to be a promising compound with drug-likeness properties, being both compliant with Lipinski's rule of five (NZ has a molecular weight of 357.4, log P of 2.157, 6 and 4 hydrogen bond acceptors & donors, respectively) and with satisfactory absorption, distribution, metabolism, excretion (ADME) traits as reported by Khusro et al. [16].

2. Methods

2.1. Plant material

Dried seeds and leaves of *MO* were purchased from the General Moringa Scientific Cooperative Society at the National Research Center (NRC), Giza, Egypt, which obtains the plant from a local cultivar. Authentication of the seeds and leaves was done by Prof. Abo Elfotouh Abdalla (taxonomy specialist and former head of Horticultural Crops Technology Department at NRC). A voucher specimen (no. 0817) of the authenticated plant was deposited at the Pharmaceutical Biology Department at the German University in Cairo (GUC), Egypt. The seeds were coarsely crushed using a laboratory blender before use and the leaves were stored untreated in a shade-dry location until use.

2.2. General experimental procedures

Solvents used for extraction and chromatographic isolation were of analytical grade. Petroleum ether, methylene chloride, chloroform, ethyl acetate, *n*-butanol and conc. sulphuric acid were purchased from El Nasr Pharmaceutical Chemical Co. (Egypt). Pre-coated TLC plates, silica gel 60 F_{254} (20 × 20 cm), silica gel 60 (35-70 mesh) for normal phase column chromatography, silica gel H, *p*-anisaldehyde, absolute ethanol and methanol were bought from Sigma-Aldrich Chemie GmbH, Steinheim (Germany). Glacial acetic acid was obtained from Sd fine-chem limited, Industrial Estate, Mumbai (India). Formic acid and HPLC-grade acetonitrile were purchased from Sigma-Aldrich Chemie GmbH, Steinheim (Germany). HPLC-grade water was obtained using Purelab UHQ water equipment, ELGA (UK).

The structure of the isolated compound was determined using collective spectroscopic techniques. Varian Nuclear Magnetic Resonance (NMR) Spectrometer was used to record ^{1}H-NMR and ^{13}C-NMR spectra (at 500 MHz and 126 MHz, respectively). The sample was dissolved in deuterated dimethyl sulfoxide (DMSO-*d6*) and tetramethylsilane (TMS) was used as an internal standard. Chemical shift values were expressed in ppm (δ) and coupling constants (*J*) in Hz. Two-Dimensional NMR spectra such as Correlation Spectroscopy (COSY), Heteronuclear Single Quantum Correlation (HSQC), Heteronuclear Multiple Bond Correlation (HMBC) and Distortionless Enhanced Polarization Transfer (DEPT-135) were also recorded. Fourier-Transform Infrared Spectrometer (Bruker Vector 22) was used to record the compound's IR spectrum. High resolution mass spectrum was recorded using a Thermo Fisher Scientific (TF, Dreieich, Germany) Q Exactive Focus system equipped with heated electrospray ionization (HESI)-II source and Xcalibur software (version 4.0.27.19). Before analysis, external mass calibration was done according to the

manufacturer's recommendations. The samples were dissolved and diluted in methanol in a concentration of 3 μM and directly injected into the Q Exactive Focus using the integrated syringe pump. Data analysis was done in positive ion mode using voltage scans and the data collection in continuous mode.

2.3. Preparation of plant extracts

The dried seeds and leaves of *MO* (700 g and 600 g, respectively) were exhaustively extracted with 70% ethanol by cold maceration. Maceration was done in 3 cycles, each for an average of 5 days. The total alcoholic extract of each plant part was then combined and separately evaporated under reduced pressure by a rotary evaporator (Buchi, G. Switzerland) at 60°C yielding a semi-solid residue of 160 g and 435 g, respectively. Each dry residue was then individually suspended in distilled water (800 and 2000 ml, respectively) and successively subjected to liquid-liquid fractionation with petroleum ether (3 × 800 and 10 × 1500 ml, respectively), methylene chloride (3 × 600 and 4 × 1400 ml, respectively), ethyl acetate (5 × 800 and 4 × 1400 ml, respectively) and *n*-butanol saturated with water (10 × 400 and 4 × 1600 ml, respectively). Each of the solvents was evaporated under reduced pressure at 60°C then the dried solvent-free successive extracts were weighed (giving 1.27, 4.84, 6.53 and 95.69 g, respectively of the seeds and 17.28, 8.18, 7.5 and 276.28 g, respectively of the leaves). The remaining water fraction of the ethanolic extract was finally concentrated to a paste under reduced pressure at 60°C and weighed 47.73 g and 122.85 g, respectively. The dried solvent-free extracts and fractions of the seeds and leaves were saved for both analytical and phytochemical investigations. Phytochemical investigation of the methylene chloride and ethyl acetate fractions of the seeds was then performed, as they were found to contain the major amounts of NZ. Chemical profiling and standardization were also done for these fractions, as well as the ethanolic seeds and leaves (which were found to also contain NZ).

2.4. Phytochemical investigation of fractions

Several solvent systems were first applied for developing the TLCs of the 2 fractions in order to decide upon the starting mobile phase of the silica columns. Such systems included: n-hexane- ethyl acetate (90:10 v/v), n-hexane- ethyl acetate (80:20 v/v), chloroform-methanol (95:5 v/v), chloroform-methanol (90:10 v/v), chloroform-methanol (80:20 v/v), ethyl acetate-methanol- water (100:16.5:13.5 v/v). The chromatograms were visualized under a UV lamp (Vilber Lourmat, Marne La Vallée (France)) (at 254 and 366 nm), before and after spraying with *p*-anisaldehyde/sulfuric acid spray reagent prepared by mixing 0.5 ml *p*-anisaldehyde with 10 ml glacial acetic acid, 85 ml methanol and 5 ml concentrated sulphuric acid. The TLC was then heated in the oven at 110°C for 2-3 mins until development of colored spots. Glass columns of different sizes were packed with silica gel adopting the wet method and silica gel H applying the dry method, and using a vacuum pump for vacuum liquid chromatography (VLC).

2.5. Standards

The reference marker compound, NZ, was isolated from *MO* seeds, purified and characterized as described previously in section 2.2. It is worthy to mention that NZ commercial standard was not purchased for comparison with our obtained calibration curve as it was not available from a trusted source. A stock of 100 μg/mL of the compound was prepared in methanol, followed by serial dilutions to give nine working solutions or the linearity study in the range of 0.05–100 μg/mL. The internal standard (IS), desipramine (DP), was purchased from Sigma-Aldrich Chemie GmbH, Steinheim (Germany) and an appropriate amount was daily freshly prepared in methanol before analysis, to finally yield a 25 μg/mL solution added to each analysis vial.

2.6. Profiling of plant extracts and fractions' components

Characterization and profiling of fractions was achieved by full scan mode measurements using Waters® ACQUITY UPLC H-Class system which consists of: Xevo™ (Triple Quadrupole Mass Spectrometer) with Electrospray Ionization (ESI) interface and MassLynx® 4.1 data acquisition software (Waters Corp., Milford, MA, USA). The mass spectrometric conditions used were positive ion mode, capillary voltage 3.5 kV, cone voltage 20V, radio frequency (RF) lens voltage 2.5 V, source temperature 500°C. Nitrogen was used as desolvation and cone gas at a flow rate of 1000 and 20 L/h,

respectively. The mass analyzer scanned from 100 to 1000 mass units. Chromatographic separation was successful on an ACQUITY BEH C18 100 mm × 2.1 mm column (particle size, 1.7 μm) (Waters, Ireland), using gradient elution from 5-100% B in 16 min, where mobile phase A was 0.1% formic acid in water and B is 0.1% formic acid in acetonitrile.

2.7. Quantification of niazimicin in seeds and leaves' extracts

2.7.1. Method development

A novel UPLC–MS/MS method was developed to quantify NZ in extracts of seeds and leaves of *MO*. The same mass spectrometer instrumentation and acquisition software as in section 2.6.were used. A linear gradient from 35-55%B in 7 min was optimized with a total run time of 11 min including reequilibration, where mobile phase A is 0.1% formic acid in H_2O and B is 0.1% formic acid in acetonitrile. The injection volume was 2 μL by partial loop injection with needle overfill. The flow rate was 0.2 mL/min using the same column described in section 2.6. at 40 °C column oven. Nitrogen was also used as desolvation and cone gas at a flow rate of 992 and 1 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 3.67×10^{-3} mbar. The optimal MS parameters were as follows: capillary and RF lens voltage as in section 2.6., source temperature 147 °C and desolvation gas temperature 498 °C. Cone voltage was 10V for both NZ and DP. The ESI source was operated in positive mode.

Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 358 > 107 with collision energy of 25V for NZ and *m/z* 266.38 > 193 (daughter ion which corresponds to the tricyclic nucleus) with collision energy of 40V for DP. Dwell time was automatically set by the acquisition software.

2.7.2. Method validation

According to the International Conference on Harmonization (ICH) guidelines [17,18], the method was validated in terms of linearity, specificity, limit of detection (LOD), limit of quantification (LOQ), precision (inter- and intra-day), accuracy, stability and robustness.

For linearity, the peak area ratio of NZ/DP was plotted versus NZ concentration to construct a linear calibration curve in the range of 0.05–100 μg/mL. Nine working standard solutions (0.05, 0.3, 1, 10, 20, 40, 60, 80, 100 μg/mL) were prepared in triplicates. The results were evaluated by least square linear regression analysis to calculate regression equation coefficient (R^2). Solutions were stored at 4ºC and their stability was tested at 0, 12, 24 and 48 hours. Specificity was defined as non-interference by other analytes.

Sensitivity of the method was determined in terms of LOD and LOQ. The LOD is the lowest concentration of NZ that the tandem mass detector can reliably differentiate from the background level at a signal-to-noise ratio of 3. The LOQ is the lowest calibration standard where precision does not exceed 15%, or where signal-to-noise ratio is 10.

To reflect the degree of repeatability of the method, precision was evaluated in terms of inter- and intra-day precision which were expressed by calculating relative standard deviation (% RSD) or coefficient of variation (CV%), where CV% = (standard deviation/mean) x 100. Quality control samples of 5 different concentrations were prepared in a similar manner as the working standards. Evaluation of the intra-day precision was done by analyzing these standards three times on the same day, whereas for the inter-day precision, analysis was repeated over 3 different days.

Accuracy was evaluated by comparing the practically measured concentration to the theoretical value of the 5 prepared concentrations of the quality control samples. The results were expressed as recovery percentage (R%) and % bias, based on five measurements at each concentration. Percentage bias represents the mean deviation from the nominal content of the sample, and is calculated as [(measured value − true value)/true value] x 100.

Matrix effect due to possible ion suppression or enhancement by the extracts was also evaluated by comparing the increase in NZ concentration in spiked ethanolic, methylene chloride and ethyl acetate fractions to that in methanol only using 3 different concentrations covering the calibration curve range (25, 60 and 80 μg/mL). Furthermore, the presence of the compound in major amounts in the extracts and the use of DP as an internal standard limited matrix effect. Percentage matrix effect was calculated as 1- (analyte signal spiked in matrix/analyte signal in solvent) x 100%.

2.7.3. Standardization of MO fractions

The developed UPLC-MS/MS method was applied to the quantitation of NZ in ethanolic, methylene chloride and ethyl acetate fractions of seeds, as well as, ethanolic and methylene chloride fraction of leaves. The compound was not present in

other fractions. Stock solutions of equal concentrations of each fraction were prepared (10 mg/mL), and diluted 100 times with methanol. All samples were filtered using 0.2 μm PTFE membrane syringe filter (Simple pure) before injection into the chromatograph.

3. Results and discussion

3.1. Niazimicin isolation from fractions of MO seeds

3.1.1. Methylene chloride fraction

A weighed amount (1.9 g) of the seeds methylene chloride fraction was chromatographed over a silica gel 60 column (40 g, 12 cm × 3.5 cm). Gradient elution with chloroform: methanol was implemented. Using 100 ml portions of mobile phase, fractions of 25 ml each were collected and monitored by TLC (using chloroform: methanol (90:10 v/v) solvent system), starting with 100% chloroform (100 ml), followed by increasing methanol by 5% (100 ml), then by 7% (100 ml). Elution continued with chloroform: methanol 95:5 (400 ml), where isolation of compound occurred yielding 0.71 g (R_f in chloroform: methanol 85:15 is 0.53 and olive-green spot visible upon spraying with *p*-anisaldehyde/sulfuric acid reagent). Spot remained single upon performing 2D TLC and with increasing polarity of the TLC mobile phase. Compound eluted with peak purity of 96.6% [Fig. S1 in Supplementary Material] by adding the areas of the peaks at 6.58, 6.69 and 6.98 min (since they showed an identical M+H peak and UV λ_{max}). The extracted compound appeared as a waxy yellowish solid. The structure of the isolated compound has been established on the basis of high-resolution mass spectrometry, UV spectral data, ^{1}H-, ^{13}C- and 2D-NMR. Compared to the previously reported isolation protocol of Faizi et al.[6], the methodology optimized in this work is much simpler, with fewer types of solvents, and less work-up steps, in addition to the exclusion of a preparative RP-HPLC step in favor of a conventional normal phase column chromatography, thus cheaper and readily-available alternative.

3.1.2. Ethyl acetate fraction

Phytochemical investigation of the seeds ethyl acetate fraction was performed, in an attempt to isolate a greater yield of the compound. A weighed amount (3.5 g) was chromatographed over vacuum liquid chromatography (VLC) column (70 g, 18 cm × 4 cm). Gradient elution starting with methylene chloride: ethyl acetate 95:5 was first used (300 ml), then increasing ethyl acetate by 5% (100 ml), followed by another 5% (700 ml). Elution continued with 5% ethyl acetate increments every 100 ml, till reaching isocratic elution with methylene chloride: ethyl acetate 35:65. NZ was present in all these fractions, which were collected in 100 ml portions, monitored by TLC and pooled together, yielding 0.52 g. NZ eluted with a purity of 45.91% [Fig. S2 in Supplementary Material] and hence was just used to complete the needed amount of the compound in a later biological study.

3.1.3. Compound identification by spectral analysis

High resolution mass [Fig. 1a] ((+)-HRESIMS) revealed a molecular-ion peak $[M+H]^+$ at *m/z* 358.1291 (calculated for $C_{16}H_{23}NO_6S$, 358.1319). UV spectrum (MeOH) [Fig. S3a in Supplementary Material] showed the compound has λ_{max} of 221($\Delta\epsilon$ + 1.56) and 244 ($\Delta\epsilon$ + 1.16) nm. FT-IR spectrum (KBr) cm^{-1} [Fig. S3b in Supplementary Material] revealed ν_{max} absorptions, characteristic for several functionalities, 3424.96 (OH or NH stretch), 2926.45 (C−H (sp^3) stretch), 1622.8 (aromatic C=C), 1513.85 (CH_2 bend), 1420.32 (aromatic C=C or C−N), 1235.18 (C−O or C=S), 831.169 cm^{-1}(substituted C=C out of plane bending). Mass fragmentation pattern as revealed by EIMS [Fig. S3c in Supplementary Material] showed characteristic fragmentation peaks at *m/z* 106 (96%), 212 (49%) and 253 (100%), which are strikingly similar to those of niazimicin, as reported by Faizi et al.[6].

^{1}H NMR spectrum [Fig. 1b] revealed the presence of α-L-rhamnose, where a downfield multiplet at δ5.35 (H1') is characteristic for the anomeric proton. All other protons in the sugar appeared also as one-proton multiplets at δ3.81 (H2'), 3.62 (H3'), 3.44 (H5') and 3.27 (H4'), except for the methyl group which appeared as a characteristic three-proton doublet at δ1.09 ($J_{6',5'}$ 6.2). In addition, the presence of three hydroxy groups was shown by a pair of doublet of doublet at δ5.04 (2′OH) and 4.74 (3′OH), with the third hydroxyl appearing as a doublet at δ 4.87, being slightly hidden by H7 doublet signal at δ4.85. Due to the fact that the compound also exists as a thiol-imine tautomer [6], another signal for the benzylic methylene appears also as a doublet at δ4.56, and consequently C7 showed two signals at δ47.53 and 47.16 [Fig. 1c]. This is confirmed by the 2D COSY plot [Fig. S4, S5 in Supplementary Material], which shows that both H7 signals couple with the NH signal at δ9.57 ($J_{NH,7}$ 6.1). This downfield resonance of the NH group shows that it is *Z* to sulfur, thus confirms that the compound is mainly the *E* rotamer, since the *Z* isomer would show an upfield NH signal at δ4.56. The thiol-imine tautomerism also affects the methylene of the

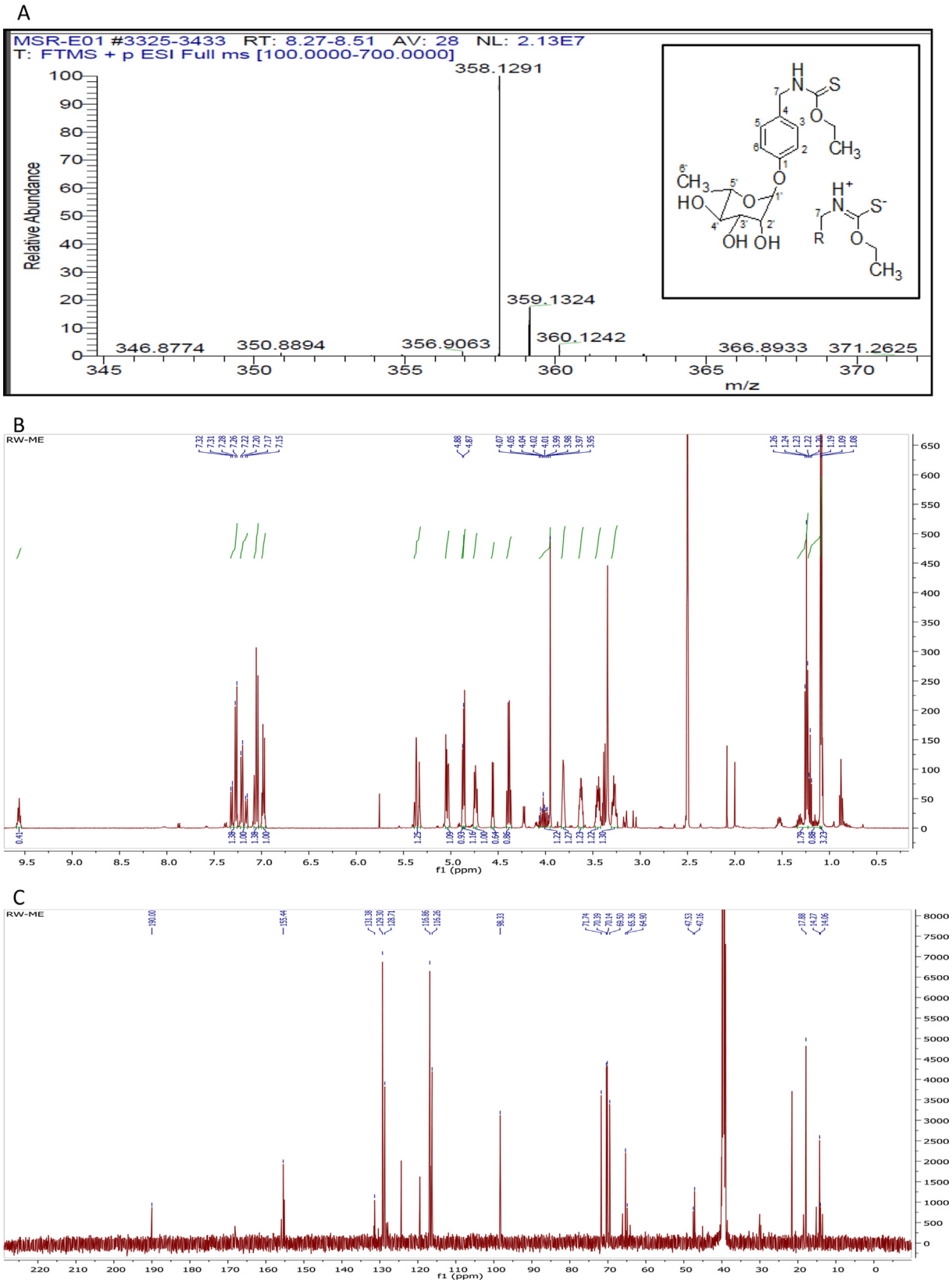

Fig. 1. Structure elucidation charts of niazimicin: (A) High resolution mass scan showing m/z of 358.1291 in the positive mode, with the structure of niazimicin (E) and its tautomer form (B) ^{1}H NMR spectrum showing all assigned protons, (C) ^{13}C NMR spectrum showing all assigned carbons.

ethoxy group, by having its protons appear also as double signals as multiplets at δ4.01 and 4.39, as confirmed by the HSQC plot [Fig. S6 and S7 in Supplementary Material]. The methyl group of the ethoxy group was similarly affected by this tautomerism, by showing its protons as a pair of triplets at δ1.24 and 1.20 and its C at δ14.27 and 14.06. Finally, the aromatic protons showed up in the characteristic aromatic region of the ^{1}H NMR spectrum (7-8 ppm), by appearing as a mutually coupled pair of doublet of doublet at δ7.29, 7.18 (H3,5) and as multiplets at δ7.05 and 6.98 (H2,6). A characteristic thiocarbonyl carbon was also observed at δ190, showing no couplings to any proton. In light of this discussion and by comparison of all spectroscopic data to previous literature [6], the isolated compound has been evaluated as (*E*)-O-ethyl-4-[(α-L-rhamnosyloxy)benzyl]-thiocarbamate, or niazimicin [Fig. 1a].

3.2. Profiling of plant extracts and fractions

UPLC/PDA/MS/MS analysis of the crude ethanolic *MO* seeds extract revealed the presence of one major compound appearing at 6.50 min [Fig. 2a]. As previously mentioned in section 3.1, this compound was specifically found in the corresponding methylene chloride fraction and appeared at 6.64 min [Fig. 2b] and in the ethyl acetate fraction [Fig. 2c], eluting at retention times (t_R) of 6.55 and 6.73 min. The same compound was also present in the leaves methylene chloride fraction and appeared at 6.50 min [Fig. 2d]. MS scan of these peaks at different cone voltages showed a molecular ion peak at *m/z* 358.10 in the positive mode, in addition to the characteristic fragments of the compound (*m/z* 106.86, 212.02 and 253.15) which is indicative of NZ. This allowed selection of the extracts and fractions for standardization.

3.3. Method Development for NZ quantification

A new UPLC–MS/MS method was developed to quantify NZ in its *MO* seeds/leaves fractions (crude ethanolic, methylene chloride and ethyl acetate). In this newly developed method, DP was used as an internal standard as it eluted with a t_R close to NZ (Δt_R = 0.11 min), hence eliminating any experimental errors occurring during quantitation due to differential noise and background computations.

3.3.1. Optimization of mass detection parameters

In quantitative UPLC/ESI-MS/MS method development, the positive ionization mode was selected because both NZ and DP have a basic amino group which is easily protonated giving a strong *m/z* response. The precursor ions $[M+H]^+$ were detected at *m/z* 358.05 for NZ and *m/z* 266.38 for DP. The capillary voltage was optimized and 3.6 kV was chosen for the analysis of both compounds due to its highest response for precursor ions. Gradual

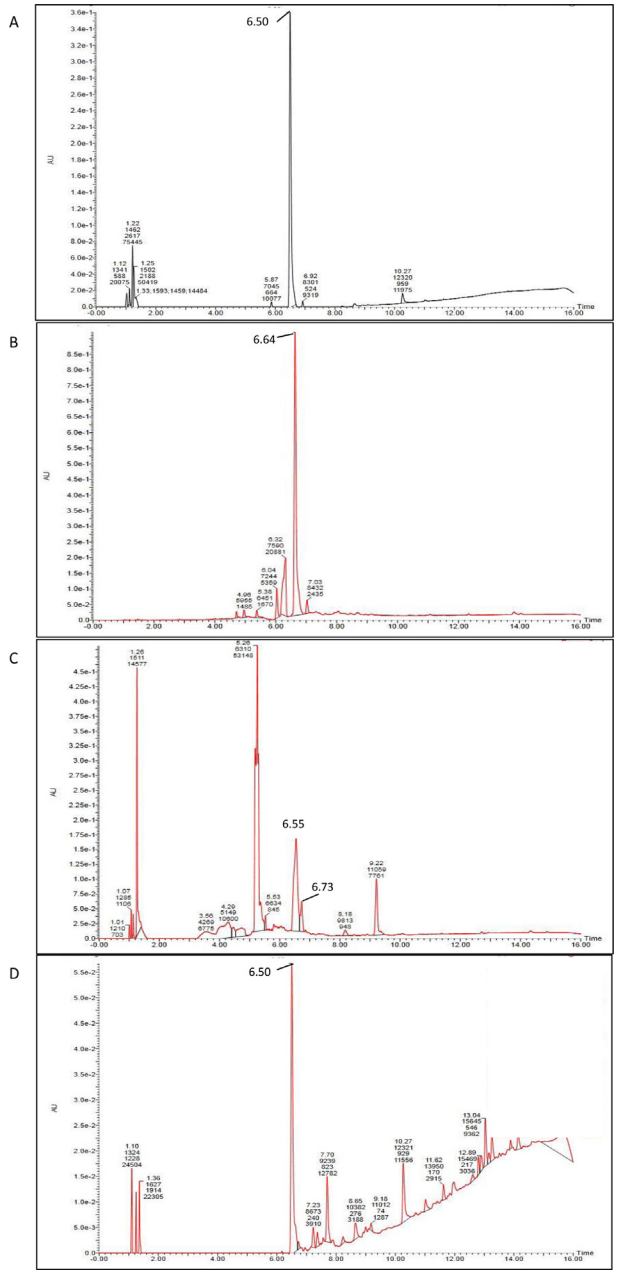

Fig. 2. Photodiode array (PDA) chromatogram of (A) seeds' ethanolic extract showing niazimicin as the major compound (t_R = 6.50 min), constituting 71% of the fraction, (B) methylene chloride fraction of the seeds showing niazimicin (t_R = 6.64 min), constituting 68% of the fraction, (C) ethyl acetate fraction of the seeds showing niazimicin (t_R = 6.55 and 6.73 min), constituting approximately 20% of the fraction, (D) methylene chloride fraction of the leaves showing niazimicin (t_R = 6.50 min), constituting 26% of the fraction.

increase of the collision energy was done to optimize the formation of product ions.

As previously described, four NZ daughter ions were detected at *m/z* 264.13, 253.15, 212.02 and 106.86 with high total ion count. The highest sensitivity was achieved by using MRM mode of 358.05 > 106.86 at collision energy of 25V, accordingly, MRM of 358.05 > 106.86 was selected to be the transition used for quantification [Fig. 3a]. Regarding the internal standard, DP, the product ion peak of the highest ion count was found at *m/z*193.04 [Fig. 3b], hence, MRM of 266.38 > 193.04 at collision energy of 40V was selected to be used for quantification [Fig. 3c].

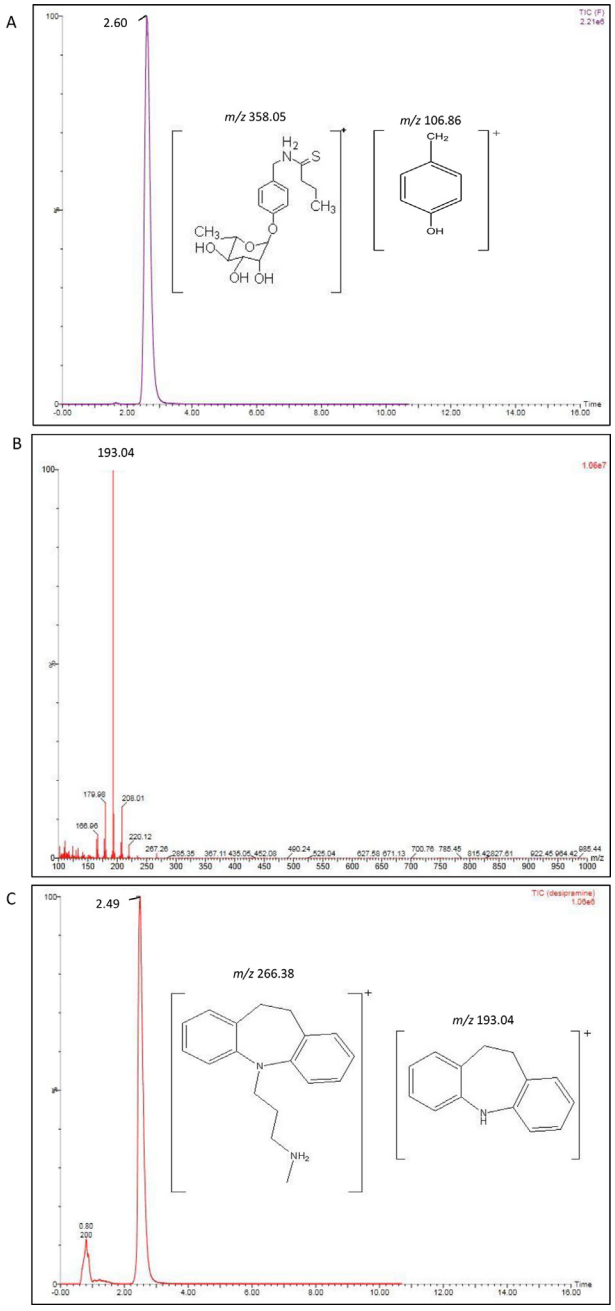

Fig. 3. (A) Multiple Reaction Monitoring (MRM) chromatogram of niazimicin at 358.05 > 106.86, (B) daughter ions of desipramine where 193.04 shows the highest intensity, (C) MRM chromatogram of desipramine at 266.38 > 193.04.

3.3.2. Optimization of LC parameters

For optimization of the LC method, an exploratory broad flat gradient run was performed from 5-100% B in 16 min, where it was observed that the peak of interest (t_R = 6.64 min) coeluted with other analytes almost midway the gradient time (t_G) [Fig. 2b]. Gradient start and end adjustments to 35-80%B in 16 min, eluted the compound of interest at an appropriate run time of 2.43 min [Fig. 4a]. However since no elution takes place at high eluent strengths, it was decided to end the gradient at weaker values, namely from 35-55%B (B: acetonitrile) in 11 min, where the peak of interest eluted at 2.42 min [Fig. 4b].

3.3.3. Method validation

The method was validated according to ICH guidelines in terms of; linearity, specificity, LOD, limit of quantification LOQ, precision (inter- and intra-day), accuracy, stability and robustness. MRM detection mode offered specificity at the selected transitions (358.05 > 106.86 for NZ and 266.38 > 193.04 for DP) where no peaks interfered with the analyte and internal standard.

Five calibration curves on three different days were constructed [Fig. 5a] where a linear calibration curve was obtained over the concentration range 0.05-100 μg/mL. Slopes, intercepts and coefficients of regression (R^2) are shown in Table 1 with %RSD <10% for both the slope and intercept.

The intra and inter-day repeatability evaluated as % RSD ranged from 3.32 to 9.96% and 1.26 to 6.84%, respectively, which is within the accepted range (<10%) [Table 2]. For accuracy, the % recovery ranged from 95.8 to 104.2% whereas % bias was lower than 5% (ranged from −4.2 to 4.2%). In addition, the method has good sensitivity with low LOD (0.02 μg/mL) and low LOQ (0.05 μg/mL). Furthermore, standard solutions of NZ in methanol (5 and 80 μg/mL) proved to be stable within 48 hours at 4°C, with %RSD of 2.5 and 1.2, respectively. In addition, the method proved to be robust upon applying deliberate small changes in flow rate, mobile phase composition, column temperature and nebulizer gas flow rate, with mean %RSD <10% in all cases. The deviations were calculated on the basis of peak areas of 40 μg/ml NZ with the default parameters as reference values. Stability of t_R of analytes in different fractions is also indicative of

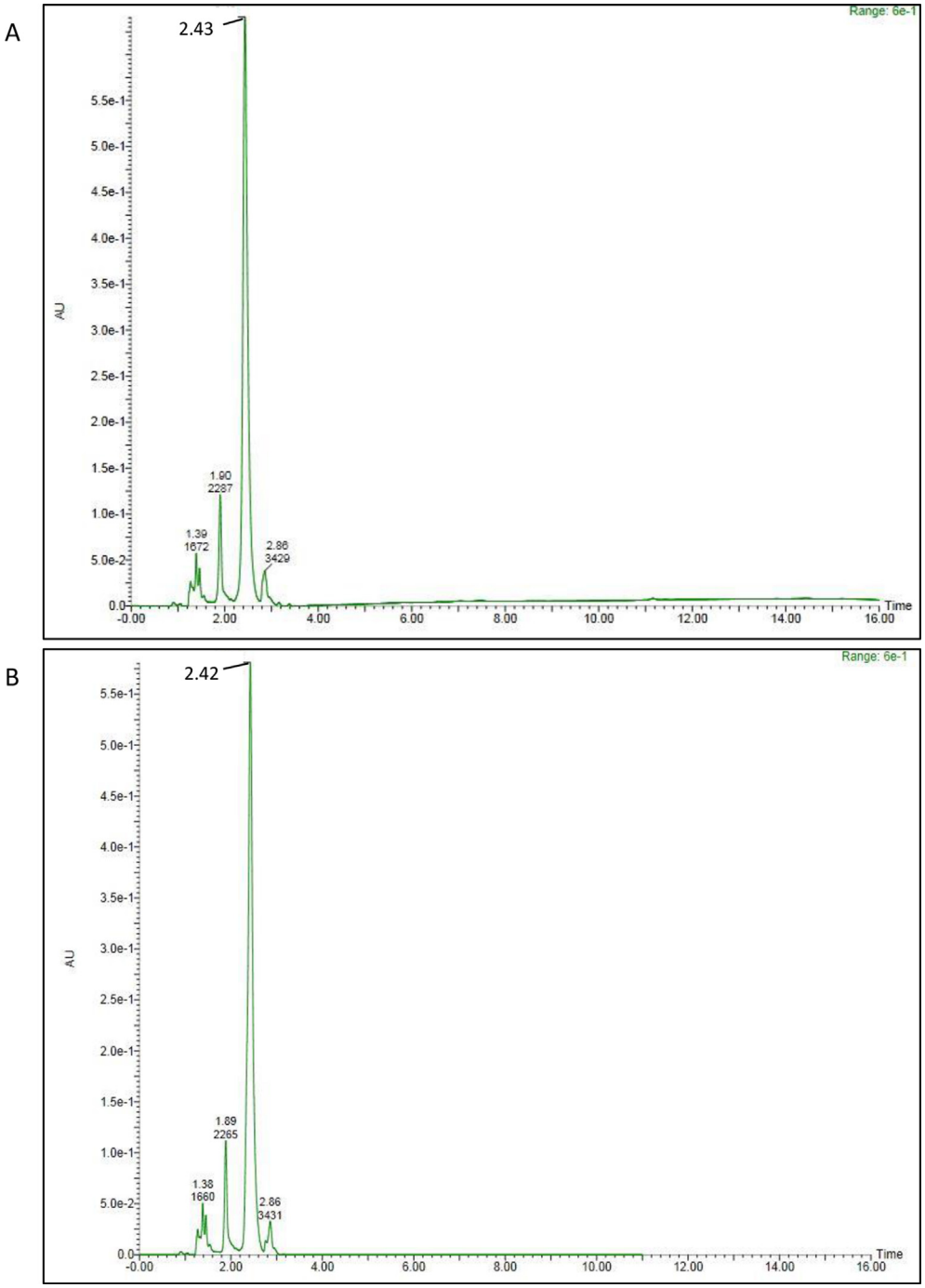

Fig. 4. PDA chromatogram of methylene chloride fraction of the seeds employing a gradient of (A) 35-80%B, (B) 35-55%B.

robustness. The percentage matrix effect at 25, 60 and 80 μg/mL in ethanolic, methylene chloride and ethyl acetate fractions was 0.5 ± 1.2%, 0.9 ± 1% and 1% ± 0.5%. As these values approach zero, this implies negligible matrix effect, where more negative values would suggest ion enhancement and more positive ones indicate ion suppression.

3.3.4. Standardization of NZ in MO seeds and leaves

NZ was standardized in extracts and fractions as per 100g of seeds or leaves. Fig. 5b illustrates an example of the seeds ethanolic extract chromatograms, where the peak areas were used for calculation of NZ content. As shown in Table 3, this extract was standardized to contain 620 mg% ± 3.2%, while its methylene chloride fraction

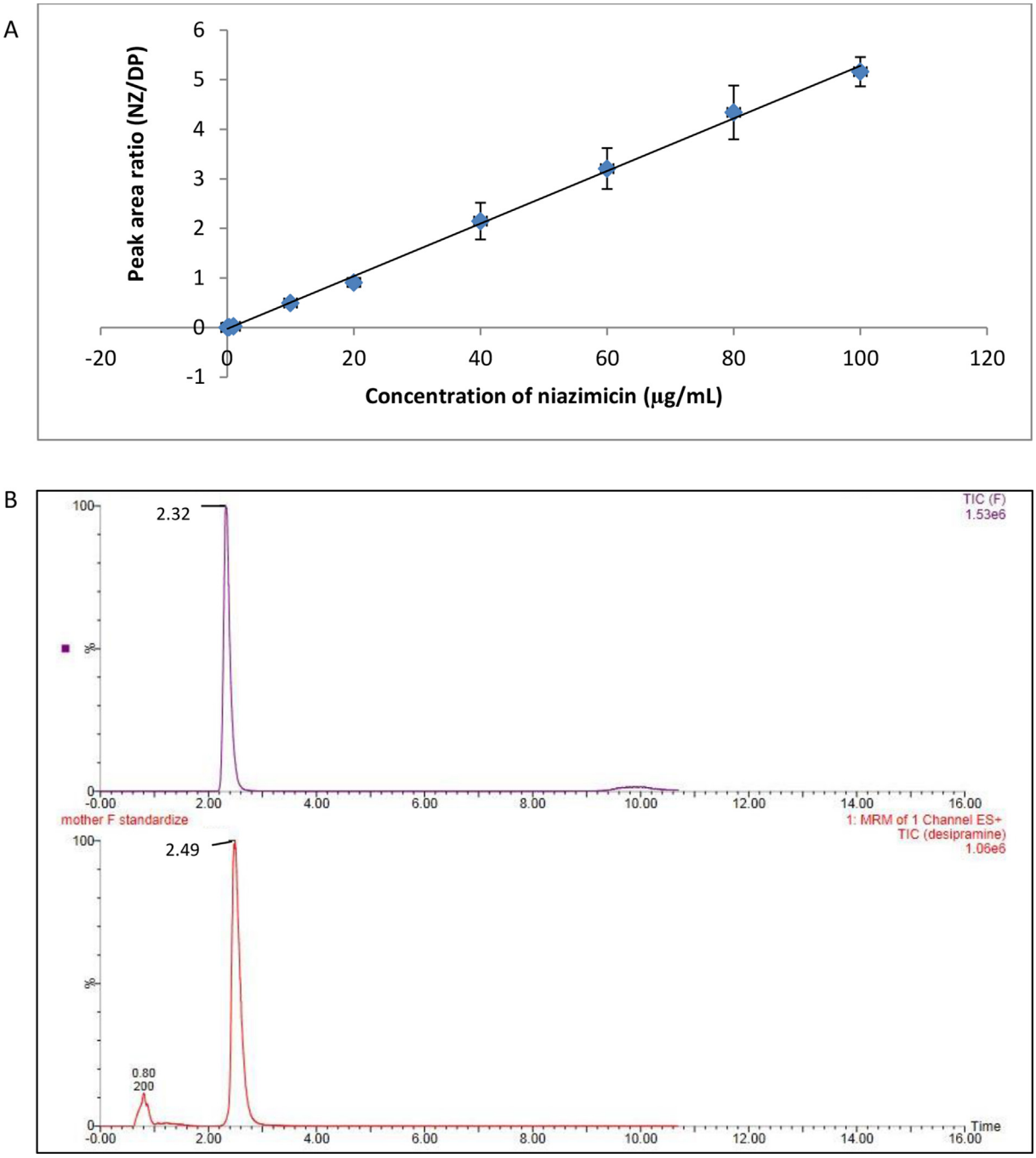

Fig. 5. (A) Mean calibration curve of niazimicin, extracted from MO over a concentration range of 0.05-100 µg/mL, (B) MRM chromatograms of niazimicin detected in ethanolic seeds extract (top) and desipramine spiked in the sample (bottom) using the optimized method conditions.

Table 1. Pre-study validation: Regression parameters for the calibration curves on three different days. One calibration curve was obtained on the first two days, and three on the third day (n = 5).

Day	1	2	3	3″	3‴	Mean	SD	%RSD
Intercept	0.023	0.020	0.021	0.022	0.019	0.021	0.002	7.530
Slope	0.050	0.057	0.052	0.051	0.053	0.053	0.003	5.140
R^2	0.998	0.997	0.985	0.995	0.997	0.994	0.005	0.540

contained three times NZ amount that was found in the ethyl acetate fraction. The leaves ethanolic extract had half the amount of the seeds' content of NZ by having 320 mg% ± 1.3%, being represented in its methylene chloride fraction. As such, we report in this work for the first time the standardization of NZ in *MO* seeds and leaves and their respective compound-containing fractions.

Table 2. Method validation parameters: precision, accuracy, and recovery.

Labeled Concentrations (μg/mL)	Measured Concentrations (μg/mL)					Precision		Accuracy	Recovery
	Day					Mean	CV (%)	Bias (%)	R%
	1	2	3	3″	3‴				
5	4.2	4.5	5.1	4.8	5.5	4.82	9.96[a], 6.84[b]	−3.6	96.4
25	25.9	22.0	24.0	23.6	24.2	23.94	8.14, 1.28	−4.2	95.8
40	41.4	37.0	45.0	43.0	42.0	41.68	9.74, 3.53	4.2	104.2
60	62.0	65.0	61.0	60.0	61.5	61.90	3.32, 1.26	3.2	103.2
80	82.9	77.0	76.8	74.1	75.5	77.26	4.39, 1.79	−3.4	96.58

[a] Intra-day precision.
[b] Inter-day precision.

Table 3. Standardization of niazimicin content in MO seeds and leaves.

MO fraction	Niazimicin Content (/100g)	
	Seeds (mg%)	Leaves (mg%)
Ethanolic	620 ± 3.2%	320 ± 1.3%,
Methylene chloride	450 ± 2.4%	300 ± 0.5%
Ethyl acetate	150 ± 1.5%	none

4. Conclusion

This is the first report of a chromatographic and a UPLC-MS-MS method for determination and quantification of NZ, a highly bioactive compound with a high potential of being a drug lead, in *MO* seeds and leaves. The isolation protocol of NZ was optimized to give a simpler procedure than what is currently reported in the literature. The novel validated analytical method proved accuracy, short analysis time, specificity and sensitivity and showed that seeds contain twice the amount of NZ as leaves. This emphasizes that the nutritional and medicinal value of NZ is significantly higher in seeds, although leaves are more commonly consumed and are the main source of NZ in all current marketed dosage forms of *MO*.

Appendix A. Supplementary Material

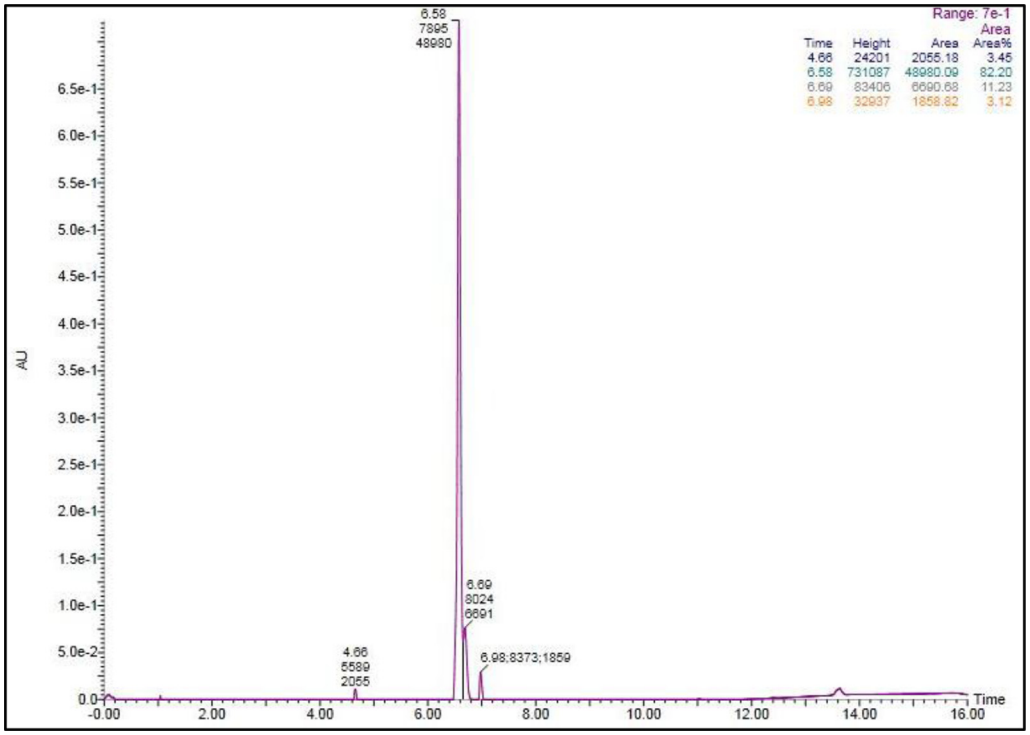

Fig. S1. Photodiode array (PDA) chromatogram of 1mg/ml of isolated niazimicin from seeds methylene chloride fraction, appearing as 3 peaks at 6.58, 6.69 and 6.98min, with a total purity of 96.6%.

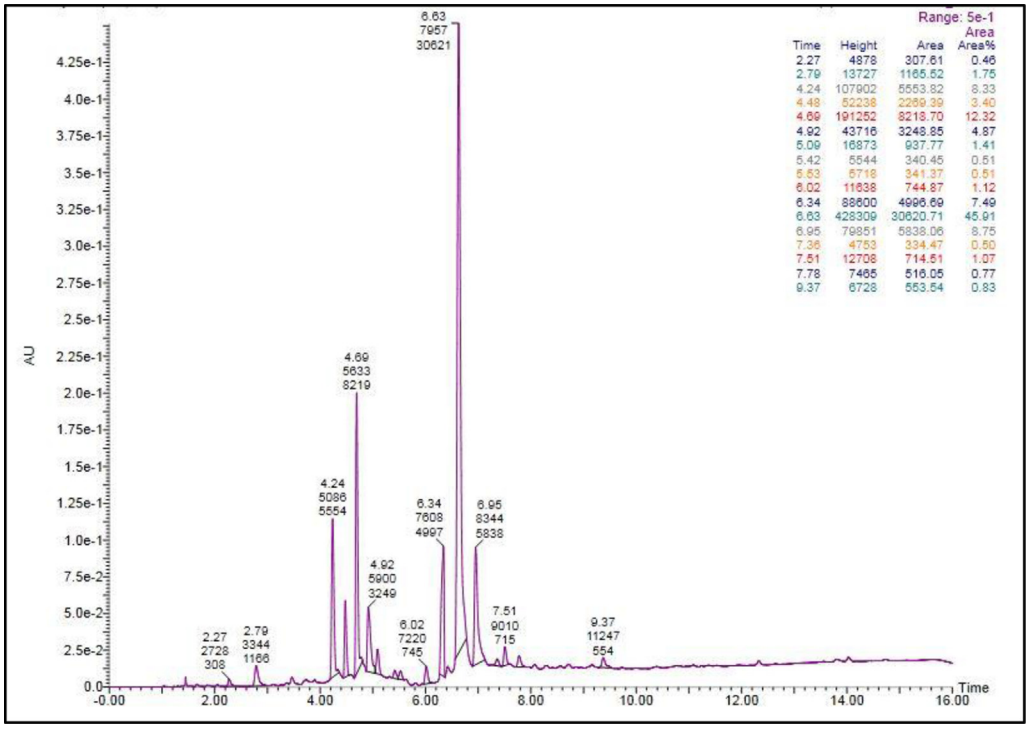

Fig. S2. Photodiode array (PDA) chromatogram of a VLC column fraction containing niazimicin (appearing at 6.63 min), with a total purity of 45.9%.

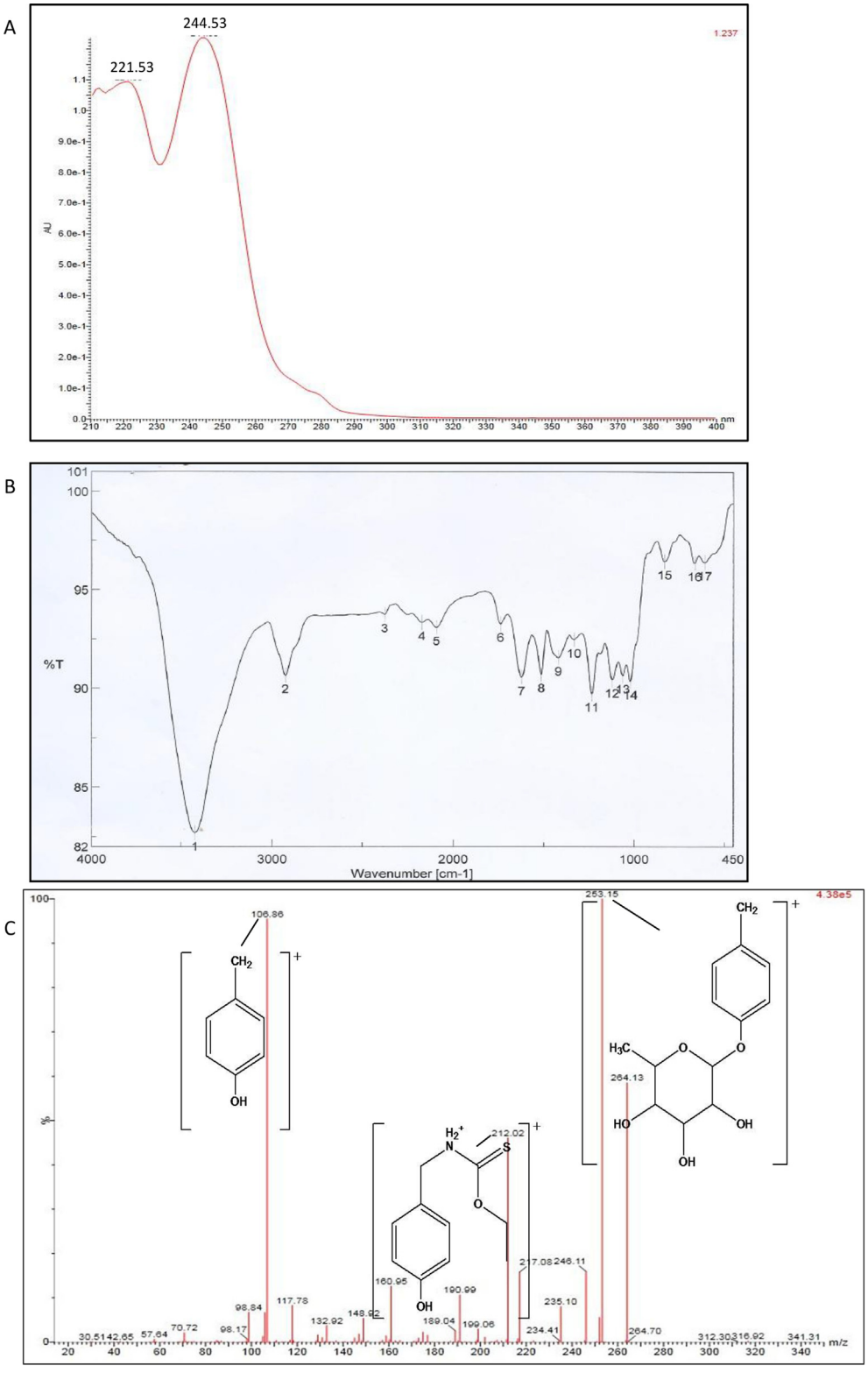

Fig. S3. UV spectrum in methanol (A), FT-IR spectrum (B) and (C) Daughter-ion scan (in the positive mode using ramped cone voltage)of niazimicin.

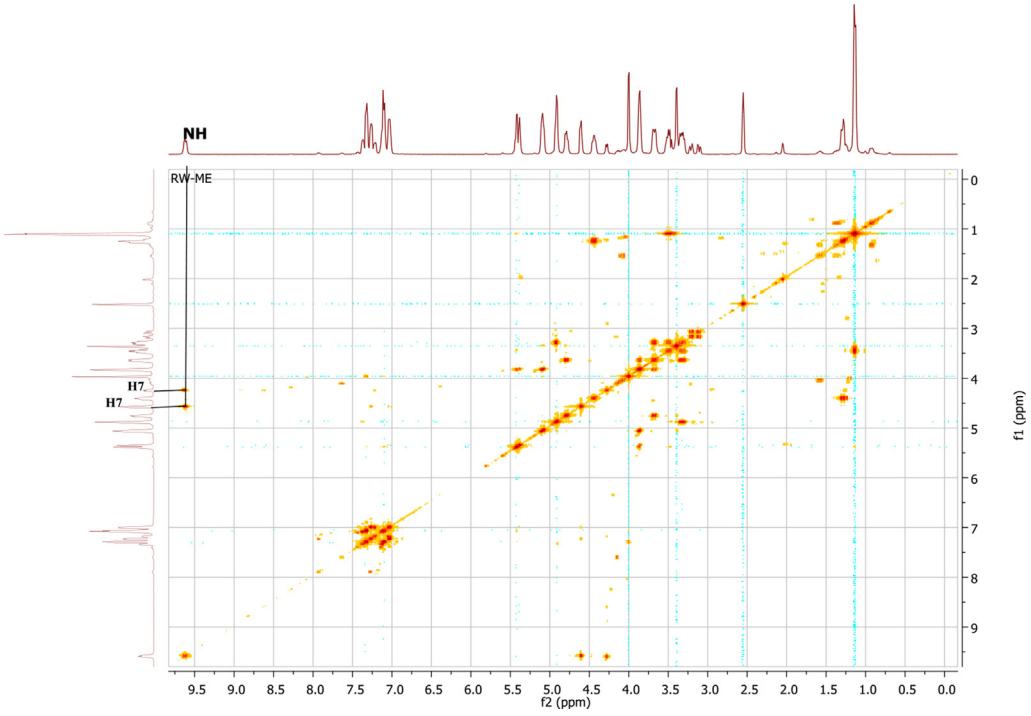

Fig. S4. Overview of 2D COSY spectrum of niazimicin showing the coupling of the protons. The coupling of NH and H7 protons is clearly shown.

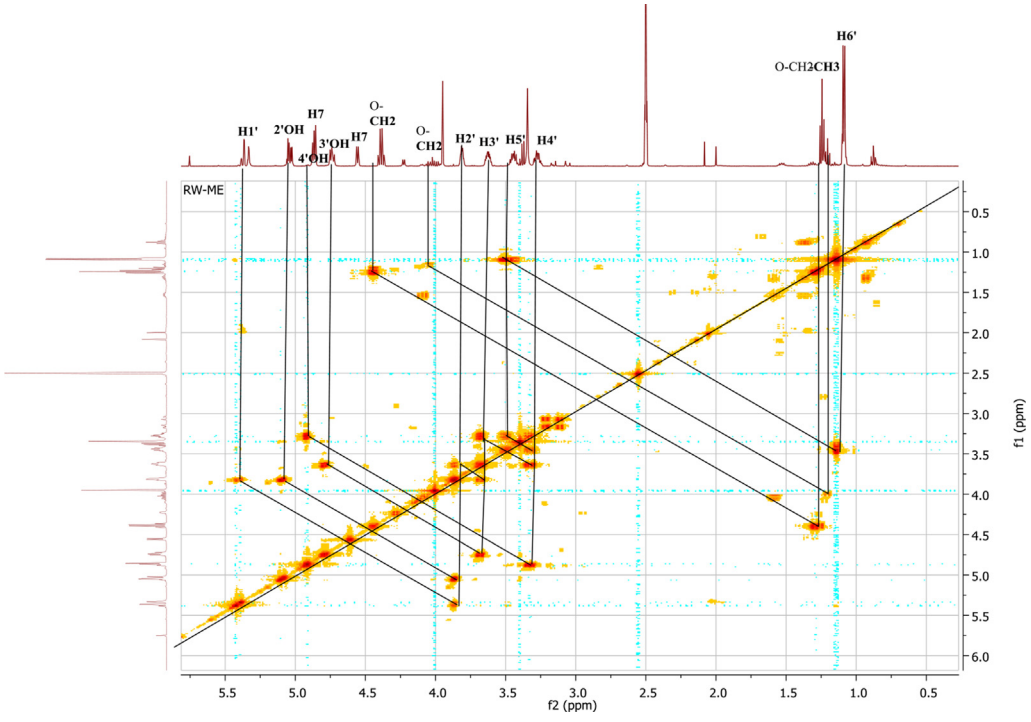

Fig. S5. Expansion of 2D COSY spectrum of niazimicin showing the major proton couplings.

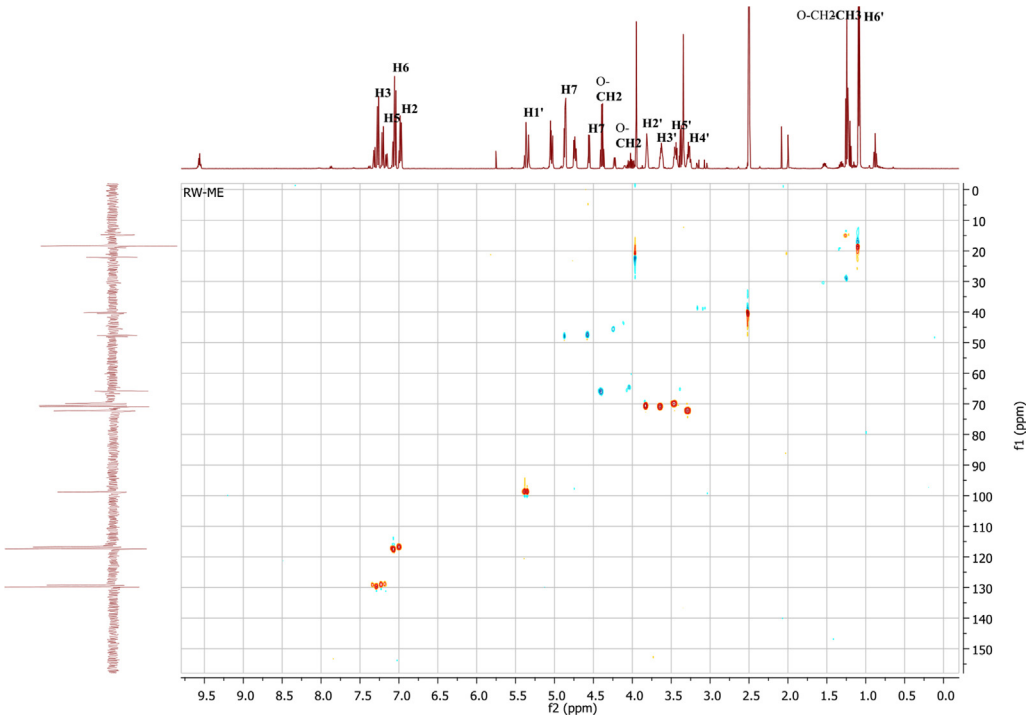

Fig. S6. Overview of 2D HSQC spectrum of niazimicin showing how the protons couple with their respective methyl, methines and methylene groups as shown by the DEPT experiment.

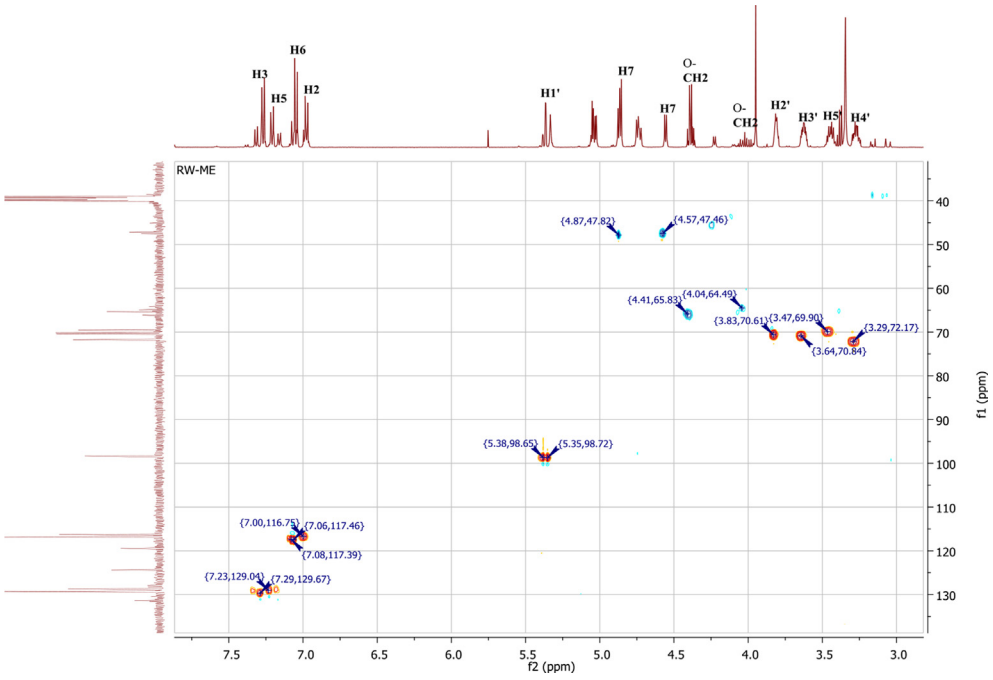

Fig. S7. Expansion of 2D HSQC spectrum of niazimicin showing the major proton-carbon couplings.

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