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Determination of bioactive nitrile glycoside(s) in drumstick (*Moringa oleifera*) by reverse phase HPLC

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Abstract

A high-performance liquid chromatographic method for the determination of novel bioactive nitrile glycosides niaziridin and niazirin in the leaves, pods and bark of *Moringa oleifera* is reported. Niaziridin is a bioenhancer for drugs and nutrients. The analytical conditions for reversed-phase HPLC with UV detection were as follows: column, Chromolith RP-18 e, 4.6×100 mm 0.5 μm (Merck); column temperature, 25 °C; mobile phase, a 20:80 (% v/v) mixture of acetonitrile: Phosphate buffer – pH 3.8; flow rate, 0.7 ml/min; detection at 220 nm. Method precision (relative standard deviation) was 1.81% for niaziridin and 1.94% for niazirin. Niaziridin (0.015% and 0.039%) and niazirin (0.038% and 0.033%) are present in leaves and pods, respectively. Niaziridin and niazirin were not detected in the bark of *M. oleifera*. Relatively higher amount of niazirin was present in leaves in comparisons to the pods while niaziridin content was about three times higher in the pods than the leaves of the *M. oleifera*. The method is robust to evaluate niaziridin and niazirin in samples from *M. oleifera* as well as for quality assurance of pharmacologically active standardized extract.
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Keywords: *Moringa oleifera*; Niaziridin; Niazirin; Rifampicin; Bioenhancer; RP-HPLC

1. Introduction

Moringa oleifera Lam. belonging to the single genus family *Moringaceae* is a small fast-growing ornamental tree originally belongs to India. Root, bark, pods and leaves of this tree are used in traditional medicine for the treatment of human diseases, whereby the leaves enriched in vitamin A and C. Pods and young leaves of the plant are primarily used for vegetative purpose (Mughal, Srivastava, & Iqbal, 1999). *M. oleifera* seed oil is also high in tocopherols (Tsaknis, Lalas, Gergis, Dourtoglou, & Spilotis, 1999). The seeds of this plant are also employed for water purification (Okuda, Baes, Nishijima, & Okada, 2001). Anti-cancer

(Guevara et al., 1999), anti-inflammatory and hepatoprotective (Kurma & Mishra, 1998a, 1998b) activities in various tissues have also been reported.

Detailed information on the nutritional value and chemical composition of *M. oleifera* seed oil is available (Ching & Mohamed, 2001; Lalas & Tsakins, 2002; Ryynänen, Lampi, Salo-Väänänen, Ollilainene, & Piironene, 2004). Number of phytochemicals various parts of *Moringa oleifera* have also been isolated (Bennett et al., 2003; Faizi et al., 1994a, 1994b; Francis, Jayaprakasam, Olson, & Nair, 2004; Lakshminarayana, Raju, Krishnakantha, & Baskaran, 2005) except the saponins which are commonly found in trees (Wina, Muetzel, & Becker, 2005). An immunoenhancing polysaccharide (Mondal, Chakraborty, Pramanik, Rout, & Islamm, 2004) and niaziminin, having structural requirement to inhibit tumor-promoter induced Epstein Barr virus activation have been

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reported from the leaves (Murakami, Kitazono, Jiwajinda, Koshimzu, & Ohigashi, 1998). Recently, a bioenhancing property of *M. oleifera* pods extract was reported and found that niaziridin rich fraction of *M. oleifera* pods enhances the bioactivity of commonly used antibiotics such as rifampicin, tetracycline and ampicillin against gram(+) and (−) bacteria and also facilitate the absorption of drugs, vitamins and nutrients through the gastro-intestinal membrane thus increasing their bio-availability (Khanuja et al., 2005). Therefore, niaziridin can be used in combination therapy with drugs and nutrients resulting in reduced drug associated toxicity, reduced cost and duration of chemotherapy.

In view of the potential bioenhancing properties of the novel nitrile glycoside niaziridin, optimization of its extraction and chromatographic conditions for rapid and precise screening of niaziridin and niazirin from *M. oleifera*, was performed. Niazirin is another bioactive nitrile glycoside belonging to *M. oleifera* (Faizi et al., 1994a, 1994b). To the best of our knowledge no validated HPLC method is available for the quantitative analysis of both niaziridin and niazirin in drumstick (*M. oleifera*). The present method is robust for the quantitative determination of niaziridin and niazirin in various tissue of drumstick. It can also be used to monitor the batch-to-batch variation of bioenhancer content in the standardized extracts of *M. oleifera*.

2. Methodology

### 2.1. Plant materials and chemicals

Drumstick (*M. oleifera*) leaves, pods and bark were collected from CIMAP research field, suburban market of Lucknow. The plant parts were air-dried and ground to powder using a mixer grinder (Philips, India).

The solvents used for extraction and chromatographic isolation were of analytical grade. Gradient grade HPLC–UV–Vis quality from Lichrosolv®, Merck, India and water purified locally on Milli-Q equipment were used. Sodium dihydrogen phosphate from Merck, India and acetic acid of HPLC quality (Spectrochem, India) and was used for pH adjustment of the eluent.
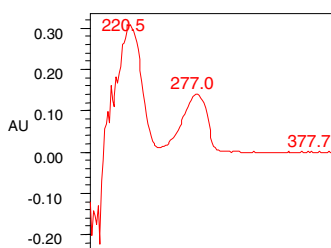
2.2. Standards

Reference marker compounds, Niaziridin and Niazirin were isolated and characterized in our lab. The purity was determined by HPLC. Spectral details of niaziridin were confirmed with our patent (Khanuja et al., 2005), while, the authenticity of niazirin was confirmed by the spectral data from the literature (Faizi et al., 1994a, 1994b). The stock solutions were prepared in methanol. The chemical structures of bioactive nitrile glycosides of *M. oleifera* are depicted in Fig. 1.
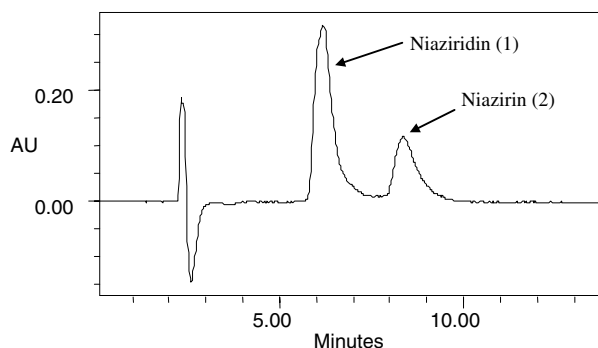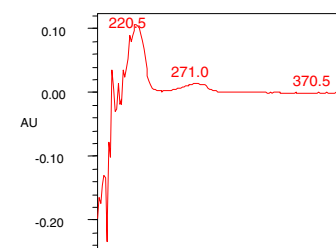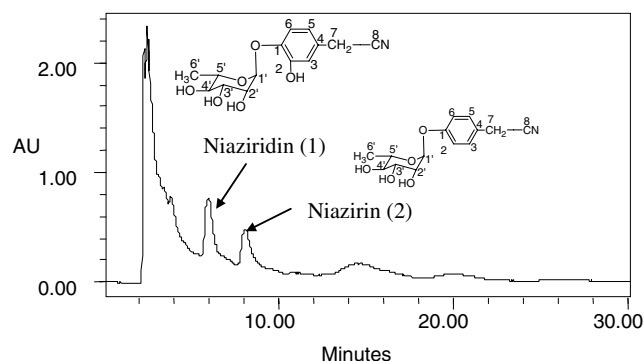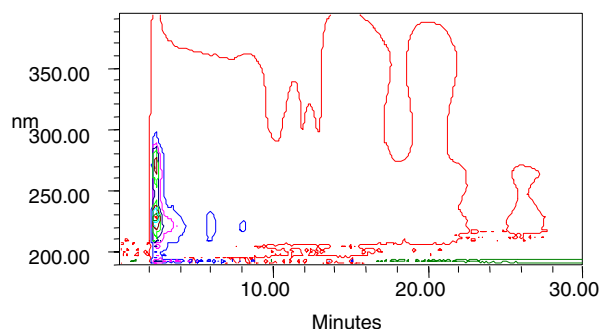

Fig. 1. Typical HPLC chromatogram of: (A) standard mixture of (1) niaziridin and (2) niazirin, (B) UV spectra of the respective peaks (C) bioactive fraction of *M. oleifera* pods analyzed by absorbance at 220 nm. UV pattern of the respective peaks and (D) iso-absorbance map (contour plot) of bioactive pod extract were indicated alongside the chromatogram.

2.3. Equipment and chromatographic conditions

The HPLC analysis have been carried out on Waters equipment: pump 600E; auto-injector-717plus; column oven; detector-996 PDA. Data acquisition and computation were carried out with Waters Melenium® software. Analyses were carried out on Merck Chromolith RP18e column, 5 μm (100 × 4.6 mm i.d.). Typical chromatograms are shown in Figs. 1 and 2.

All of the standards and extracts were filtered through a 0.45 μm syringe membrane filter (Type Millipore) and analyzed by HPLC. Analysis was carried out at 25 °C on a Chromolith RP18e (Merck) column. Prior to use, solvents were filtered with 0.45 μm, 50 mm diameter membrane filter (Millipore) and sonicated for 15 min in a Micro clean −109 bath (Oscar, India). Chromatography was carried out using mobile phase of methanol–sodium dihydrogen phosphate–acetic acid buffer (0.1 M, pH 3.8) (20:80) (% v/v). The flow rate during the analysis was 0.7 ml/min and peaks were detected at 220 nm. Although detection at 275 nm was more selective but detection at 220 nm was more sensitive and did not pose a problem in terms of co-elution with impurities. Therefore, quantitation of niaziridin and niazirin was performed at 220 nm peak area data.

2.4. Procedure

2.4.1. Extraction of M. oleifera tissues

The dried powdered leaves, pods and bark (2 g each) of *M. oleifera* were extracted with 25 ml of various combinations of ethanol–aqueous solution (0%, 20%, 40%, 60%, 80%, 100%, v/v) by incubation at 30 °C for 24 h. Each extract was filtered and solvent removed under vacuum at 40 °C. The residue was re-dissolved in 3 ml of HPLC grade methanol. Niaziridin and niazirin in various extracts were analyzed by an analytical HPLC system. It was observed that the extractability of niaziridin was greatly influenced by the ratio of extracting solvent mixtures i.e. ethanol–water. The most effective solvent for the maximum extraction of niaziridin and niazirin was 40% ethanol in water for pods and 60% ethanol in water for leaves (Fig. 3). Both the markers were not extracted from bark sample under experiment conditions studied. Thus we
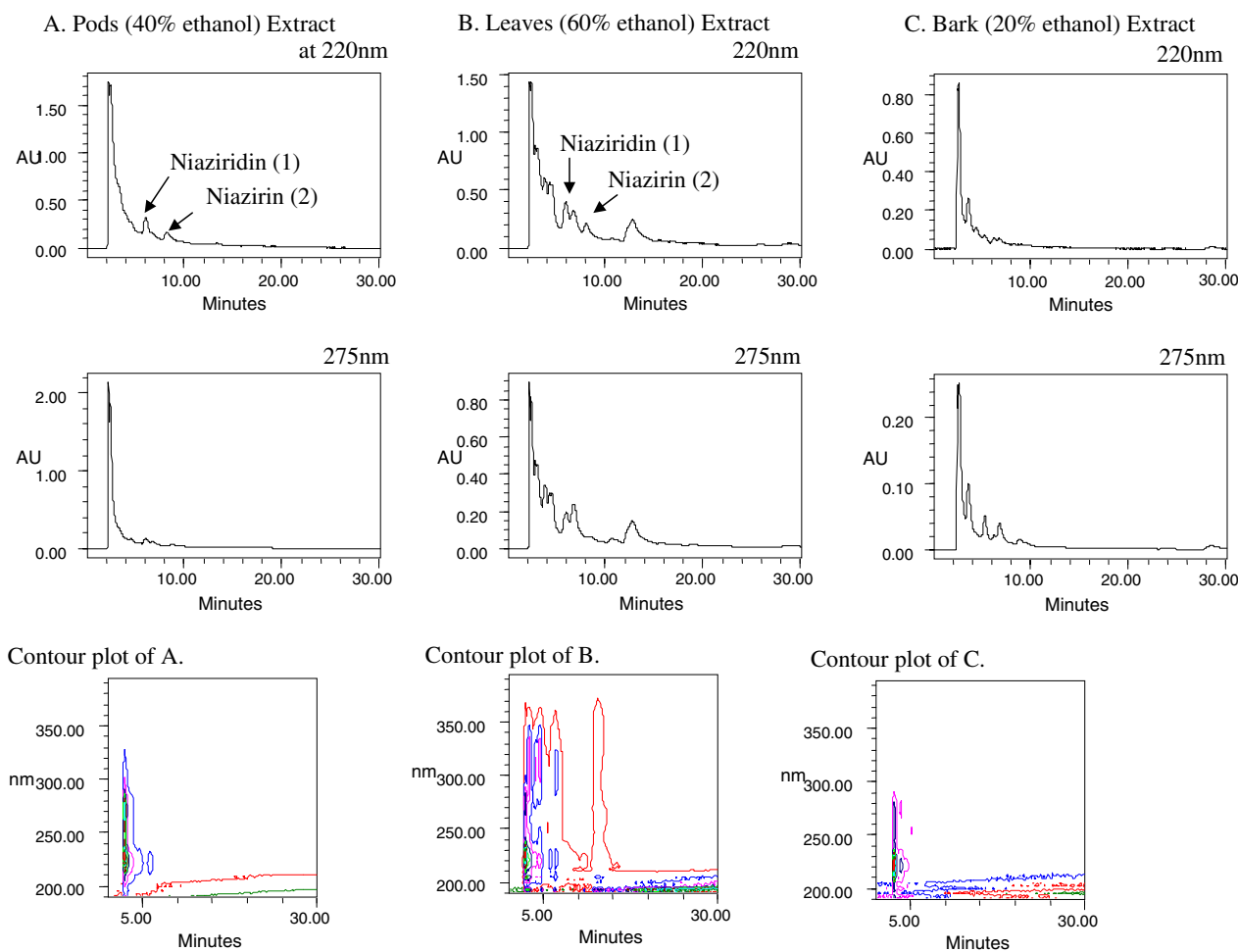

Fig. 2. HPLC profile of: (A) pods, (B) leaves and (C) bark of *M. oleifera* analyzed by absorbance at 220 nm and 275 nm, contour plot of HPLC pattern of: (A) pods, (B) leaves and (C) bark of *M. oleifera* UV absorbance at 190–400 nm, respectively.
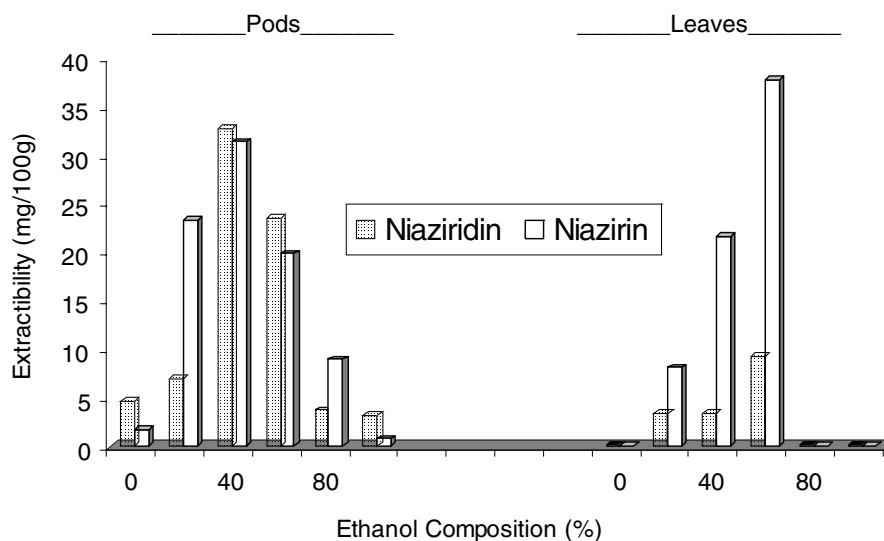
Fig. 3. Extraction of niaziridin and niazirin with various concentrations of ethanol–aqueous composition for pods and leaves of *M. oleifera*.

selected 40% ethanol–water solution as extracting solvent for pod in our further study. In order to optimize other extracting conditions, we further carried out an experiment on temperature and time. Extractability (mg/100 g) of niaziridin and niazirin from *M. oleifera* was observed at $40, 60, 90,$ and $120\,°C$ for $60, 90, 120, 150,$ and 180 min, respectively. Result shows that extraction of pods at $60\,°C$ for 120 min was the ideal for maximum extraction of 33 mg/100 g and 32 mg/100 g of the target markers i.e. niaziridin and niazirin, respectively.

2.4.2. Preparation of standardized extract of M. oleifera pods

Ethanol–water extract of *M. oleifera* pods was lyophilized and re-dissolved in water and filtered. The insoluble portion was dissolved in methanol. The water soluble portion was successively extracted with hexane, chloroform, ethyl acetate and *n*-butanol, respectively. Na_2SO_4 (anhydrous) was added to each organic fraction for removing the moisture. All the solvent fractions were concentrated at $40\,°C$ under reduced pressure and tested for bioenhancing activity of rifampicin. The results indicated that ethyl acetate fraction obtained from the crude pod extract possessed the activity enhancement property. The fraction with maximum bioenhancing activity was termed as pharmacologically active standardized extract. We further confirmed that the HPLC method developed by us is able to ensure the efficacy and quality of standardized extract.

2.5. Statistical analysis

Data were processed and recorded as means ± standard deviation of triplicate measurements. Analyses of variance (ANOVA), significance differences between the means and least square regression (Steele & Torrie, 1980) were performed using Graph PAD Prism version 3.0 for Windows, GraphPad Software, San Diego, CA.

3. Result and discussion

3.1. Separation and detection of niaziridin and niazirin

Separation and photodiode array spectrum of (A) standard compounds niaziridin (**1**) and niazirin (**2**) with respective UV-spectra, (B) representative HPLC chromatograms of bioenhancer enriched active fraction of pods with their iso-absorbance map (contour plot) using Chromolith RP18e column are presented in Figs. 1 and 2 show the HPLC profiles of regenerable tissues, pods, leaves and bark by multiple monitor (220 and 275 nm) and contour plot (190–400 nm) using a photo-diode array detector. The targeted compounds were not detected in bark sample in studied combinations of ethanol–water. The contour plot of the UV absorbance intensity of the compounds shows both the compounds, which have detectable UV absorbance in the extracts of various parts of the plant. The presence of niaziridin and niazirin in various plant parts was confirmed by comparison with the retention time and with the UV spectra matching of the compound in respective extract of the crude drug (Fig. 2).

The content (%) of bioenhancer constituent niaziridin and niazirin in various vegetative and regenerable tissues viz. leaves, pods and bark of drumstick were studied for research field station and local market collected samples. Leaves and pod sample of research filed station showed niaziridin (0.015 ± 0.005) and (0.039 ± 0.006), respectively where as niazirin was observed (0.038 ± 0.004) and (0.033 ± 0.008), respectively. Market collected sample showed (%) content of (0.010 ± 0.008) and (0.031 ± 0.006) for niaziridin and (0.038 ± 0.010) and (0.029 ± 0.010) for niazirin, respectively. Niaziridin and niazirin were not detected in the bark samples. Niazirin was present in slightly higher amount in leaves in comparisons to the pods while niaziridin content was three times higher in the pods than the leaves. The content of niaziridin in market sample was

lower than the plant samples of research filed station. This stresses the importance of chemical analysis in discriminating the plant tissues for the isolation of bioactive constituents. From the above results, it is evident that for the isolation of niaziridin, pods of *M. oleifera* while for niazirin leaves may be used as potential source. Since extracts were made immediately after drying of the plant material and extracts were stored at 4 °C, decomposition of bioenhancer compounds can be ruled out in the sample.

3.2. LC method validation

Several analytical parameters were evaluated to validate the new LC method using recommendations defined by ICH guidelines for method validation (ICH, 1994, 1996).

3.2.1. Calibration graphs, limits of detection/quantification (LOD/LOQ)

Standards containing methanolic mixtures of **1** and **2** were prepared by dilution using 1 mg/mL stock solution. Five standard of working solutions were prepared by MeOH dilution with concentrations within the expected range of concentrations in the material under investigation. The calibration curves were determined using the least-squares method, for independent variable (X) the concentration (mg/mL) and for dependent variable (Y) the peak area of niaziridin and niazirin, respectively was used. Regression analyses of both the compounds were performed by Graph PAD Prism 3.0. Linear regression parameters slope = 1.99×10^7 and 7.37×10^6; Y-intercept = -1.66×10^5 and -1.29×10^5; X-intercept = $8.37 \ 10^{-3}$ and 1.76×10^{-2}; $S_{y,x}$ at 95% confidence intervals = 2.09×10^4 and 8.5×10^3; correlation coefficient $R^2 = 0.9992$ and 0.9991 were for niaziridin and niazirin, respectively. The curves confirm the significant linear relationship between the concentration and the peak area.

LOD and LOQ were determined using the linear regression equation. Following equations were applied: LOD = $3S_{y,x}/b$ and LOQ = $10S_{y,x}/b$, where $S_{y,x}$ is the standard deviation of the Y-value distribution around the regression line and b is the slope of the calibration curve. Applying the equations, we determined LODs for niaziridin and niazirin to be 0.003 and 0.004 mg/mL, respectively and LOQs were in the same way determined as 0.011 and 0.012 mg/mL, respectively.

3.2.2. Selectivity/specificity

A diode array detector was applied for the selectivity test. The test was performed on a niaziridin and niazirin in extract of *M. oleifera* Pods. The test was carried out using peak purity judgment, which is part of the Waters Millennium® HPLC-PDA system for handling 3D chromatographic data. The principle of peak purity judgment is to obtain purity angle and purity threshold, of individual peaks. The reliability of the peak purity test can further be enhanced by carrying out the test at various wavelengths.

Typically, when using Waters Millennium® PDA software, a component with a peak purity angle lower than its purity threshold would be considered most likely to be a pure substance and the separation would be acceptable. The absorption spectra of both peaks (1 & 2) were stored in the software. The peaks associated with niaziridin and niazirin in the extracts were identified by retention times (5.953 and 8.085 respectively) and spectra between 200 and 400 nm compared with the library of reference compounds stored in the software. It was shown that in real sample analysis i.e. extract of *M. oleifera* the peak purity angle (niaziridin −0.780 and niazirin −1.981) was lower than peak threshold (niaziridin −1.630 and niazirin −2.187).

It is evident that at 220 nm, the degree of peak purity is higher and the absorption spectrum of both compounds was maximum. Quantitation of compounds both in reference and sample solution was performed at 220 nm peak area data acquisition. The selectivity has been shown on a chromatogram for one of the sample of *M. oleifera* pods (Fig. 1).

3.2.3. Recovery

Three different concentrations diluted from the stock solution were added to an extract with a known content of niaziridin and niazirin and the recovery of respective constituent was calculated. The recovery (R) was calculated as $R = (C_{found} - C_{sample})/C_{added}$, where C_{found} is the concentration in spiked sample, C_{sample} is the concentration in the sample prior to spiking and C_{added} is the concentration of the added standard. Three concentrations of standard compounds added in the range of 100–120, 180–200 and 240–260 μg/mL were used during the study. The results of the tests were acceptable, as the average recovery of niaziridin and niazirin were 86% and 95%, respectively.

3.2.4. Precision

Precision is a test for the distribution of concentrations measured. Two different concentrations of the stock solutions were exposed to six determinations on the same day. Repeatability of analysis was performed in three concentration range from 0.4 to 0.9 mg/mL and 0.8–1.8 mg/mL was used during the experiment for niaziridin and niazirin, respectively. All RSD values were below 2%, which is considered to be acceptable.

3.2.5. Intermediate precision

This test was carried out using the same equipment on different days for three different samples run in triplicate. All RSD values obtained for both niaziridin and niazirin were below 5% in the acceptable range of analysis.

3.2.6. Robustness

Robustness of the method was tested by varying two parameters viz. flow rate and column oven temperature. One parameter was kept constant during variation in other parameter. Different flow rates (0.5, 0.7 and 1.0 mL/min) and column oven temperatures (25, 27, and 29 °C) were

used to perform this test. From previous experience it was known that the eluent pH should be <4.0. A pod extract with known content of niaziridin and niazirin was used for this test. The deviations were calculated on the basis of peak areas with parameters 0.7 mL/min and 27 °C as reference values, which is, the middle value of flow rate and temperature respectively. Mean deviation is <5% which is acceptable for analysis.

3.3. Optimization of extraction condition

Extractions were carried out in triplicate using 40% ethanol–aqueous for pods. This crude extract was further enriched for marker compounds by extracting with ethyl acetate for maximum bioenhancing activity. The extraction efficiency (Extractability ± SD) was dependent on extraction time and temperature. High variation in extractability of niaziridin (32.84 ± 8.38 and 32.90 ± 4.23) and niazirin (31.42 ± 4.19 and 31.75 ± 2.22) at initial time i.e. 60 and 90 min was observed. In order to ensure complete extraction, extraction was carried out at 60 °C for 120 min, and the standard deviations for triplicate analysis are within an acceptable limit 33.05 ± 1.32 and 32.04 ± 1.09 for niaziridin and niazirin, respectively.

3.4. HPLC finger-print of Moringa oleifera active fraction

The peaks of niaziridin and niazirin from pods of *M. oleifera* were detected at retention times 5.953 and 8.085, respectively on of the contour plot in Fig. 1A and C. Thus, in addition to the above testing whether the extract have the pharmacological activity of bio-enhancer or not, this HPLC pattern analysis, the so-called "fingerprint" method, could provide a useful means of identifying the crude drugs and preparing batches of pharmacologically active standardized extract. Although, numerous compounds which do not possess UV absorbance such as polysaccharide and peptides can not be detected by this method, the fingerprint similarity of the batches may be primarily useful in assessing the homogeneity of the active fraction, which should lead to constant efficacy.

The efficacy of the bioenhancer enriched fraction of pod extract was evaluated for bio-enhancing effect under in vivo conditions. Oral administration of the bioenhancer enriched fraction 1 mg/kg in combination with rifampicin 25 mg/kg body weight significantly enhanced the bioavailability of rifampicin when compared with the serum of mice administered only with rifampicin. Two independent batches were prepared using the same pod of *M. oleifera* crude drugs by the same procedure. The fingerprint analysis of the two batches of active fraction of *M. oleifera* showed similar HPLC profiles to that in Fig. 1C.

4. Conclusion

The LC profile of pods, leaves and bark was different with respect to the content of analytes. It can be concluded that because of simple extraction procedure, short analysis time and high accuracy, the method can be a useful tool for screening of bioenhancer nitrile glycoside(s) **1 & 2** in regenerable tissues of *M. oleifera* and in assuring the quality of active standardized extract.

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