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(*Special Theme: Women in Chemistry*)

Recycling HPLC for the Purification of Bis-Styryllactones from the Stembark of *Goniothalamus lanceolatus* Miq.

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An endemic plant from the rainforest of Sarawak, Malaysia, *Goniothalamus lanceolatus* Miq. was chosen for a comprehensive phytochemical study because of its ethnomedicinal properties amongst the natives. Isolation and purification of minor compounds having similar retention characteristics were made possible by utilization of preparative recycling HPLC. This approach allows the unresolved peaks of mixtures to recirculate into the column due to a recycle valve in the HPLC system, until the peaks were successfully separated as pure entities. Recycling HPLC expediate the isolation and purification of complex mixtures while minimizing the cost of the research. This paper discussed the approach of isolating minor components with a very low yield using recycling HPLC. A total of four new bis-styryllactones, goniolanceolatin B, goniolanceolatin C, goniolanceolatin F and goniolanceolatin H, and three styryllactones, (6*S*,7*S*,8*S*)-goniodiol-7-monoacetate, (1*S*,5*S*,7*R*,8*S*)-3-exo,7-endo-(+)-8-epi-9-deoxygoniopypyrone and (1*S*,5*S*,7*S*,8*S*)-(-)-goniofupyrone B were successfully isolated.

Keywords: Recycling HPLC; *Goniothalamus*; *Goniothalamus lanceolatus*

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The genus *Goniothalamus* (Blume) Hook. f. & Thomson consists of flowering plants and is one of the largest genera in the family Annonaceae, with about 165 species comprising of shrubs, and small to large trees [1]. The species can be found in tropical forests of South-east Asia, throughout Indochina and Malaysia, and are widely distributed in lowland and submontane areas [1,2]. In Malaysia, the genus *Goniothalamus* is found in both peninsular and eastern Malaysia. According to Aslam et al. (2016) [3], there are about 50 species of *Goniothalamus* in Malaysia distributed in lowland forests and very few mountain species. *Goniothalamus* species are used in traditional medicine to treat illnesses ranging from post-partum medicine, abortifacients, remedies for fever, stomachache, rheumatism, edema, asthma, malaria, cholera, and insect repellents [4]. Two definite classes of secondary metabolites commonly isolated from this genus are styryllactones and acetogenins, which have been reported to have pharmacological activities against numerous diseases and cancer cell lines [1].

Goniothalamus lanceolatus Miq. is an indigenous plant of East Malaysia. This plant is used by the natives as a traditional medicine to treat cancer, fever, and skin diseases. It is also utilized as mosquito repellent. Previously, our group reported the phyto-chemical study of the crude dichloromethane extract from the stembark of *G. lanceolatus* Miq. has resulted in the isolation of 7 new bis-styryllactones and 12 styryllactones [5,6]. Due to the similarity of their skeleton and different stereocenters or location of functional groups, separation using conventional methods such as open column, gravity chromatography was tedious, time consuming and required a high volume of solvents. Some of the compounds were difficult to separate from their complex mixtures, and hence, the need for effective and innovative separation techniques. According to a review by Sidana and Joshi (2013) [7], recycling in an HPLC system has been used for the isolation and purification of different types of natural products including enantiomers, diastereomers, epimers, positional isomers, and structurally related or unrelated compounds with similar retention characteristics [8–14]. Recycling HPLC increases separation efficiency by incorporating a recycle valve in the HPLC system to recirculate the unresolved peaks back to the column, and no fresh solvent is required during the recycle phase, making the method economical and practical.

In the case of isolation of compounds from the dichloromethane extract of the stembark of *G. lanceolatus* Miq., the closed loop recycling technique was used, wherein the mixtures were recycled in an isocratic mode, using reversed phase silica support, to achieve the desired separation of the components. Seven compounds were successfully purified in a minute amount using recycling HPLC, namely goniolanceolatin H **1**, (6*S*,7*S*,8*S*)-goniodiol-7-mono

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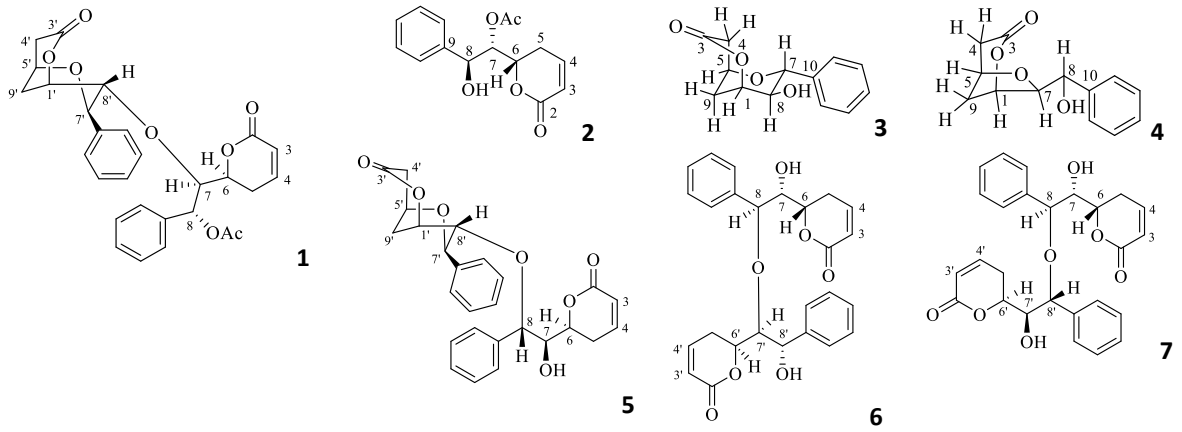

Figure 1. The structures of purified styryllactones and bis-styryllactones [5,6]

acetate **2**, (1*S*,5*S*,7*R*,8*S*)-3-*exo*,7-*endo*-(+)-8-*epi*-9-deoxy goniopypyrone **3**, (1*S*,5*S*,7*S*,8*S*)-(-)-goniofupyrone B **4**, goniolanceolatin F **5**, goniolanceolatin C **6** and goniolanceolatin B **7**. Compounds **1**, **5, 6** and **7** were new bis-styryllactones isolated from the genus. The structures of the isolates and their absolute stereo-chemistry are shown in Figure 1. The present paper discuss the setup of recycling HPLC, its applications in the separation of complex mixtures, and its ability to isolate minor compounds in minute amounts, which is not possible with conventional methods and even semi-preparative HPLC.

EXPERIMENTAL

Plant Materials and Chemicals

The stembark of *G. lanceolatus* Miq. was collected in Lundu, Sarawak, Malaysia in June 2012. It was identified by the late Prof Dr. Kamaruddin Mat Salleh (Botany Department, Faculty of Science and Technology, Universiti Kebangsaan Malaysia). The voucher specimen (FBAUMS 108) was deposited in herbarium of the Universiti Malaysia Sarawak before being couriered to Atta-ur-Rahman Institute for Natural Product Discovery, Universiti Teknologi MARA Selangor Branch, Bandar Puncak Alam, Selangor, Malaysia.

Extraction work for the crude extracts of the stembark was done by using analytical grade solvents of hexane, dichloromethane, and methanol (Sigma Aldrich, St. Louis, Missouri, USA). For fractionation by medium-pressure liquid chromatography (MPLC), the analytical solvents used were hexane, ethyl acetate and methanol. Methanol and acetonitrile were of HPLC grade (Sigma Aldrich, St. Louis, Missouri, USA). Ultrapure water (18 MΩ/cm) was obtained from a PURELAB® Option water purification system (ELGA), from Veolia Water Technologies, Paris, France.

Instruments

Fractionation of the dichloromethane crude extract was carried out using MPLC on a Yamazen Flash Liquid Chromatography W-Prep 2XY instrument, with a 120 g silica gel 40 µm (46 × 130 cm) and a 250 g silica gel 40 µm (46 × 180 cm) column. Thin layer chromatography (TLC) was performed on NP F254 plates (20 cm × 20 cm) from Merck (Darmstadt, Germany). Analytical HPLC was performed on an Agilent 1100 Series HPLC system, equipped with a Diode Array Detector 1200 series (G1315B), Micro degasser (G1379A), and a quaternary pump (G1311A) with a C18 reversed phase column (Sunfire 5 µm, 4.6 × 250 mm). Semi-preparative HPLC was done using an Agilent 1200 Series Binary Pump system, equipped with a Multiple Wavelength Detector (G1364B) with a C18 reversed phase column (Sunfire 5 µm, 10 × 250 mm). Purification of compounds were done using JAI (Japan Analytical Industry) recycling HPLC (LC-9103) paired with Diode Array and using Jaigel-ODS-AP, SP-120−15 (20 × 250 mm) column. NMR spectra were measured on Bruker Avance 600 FT-NMR (Billerica, MA, USA), in deuterated chloroform without TMS.

Extraction and Fractionation

Plant extractions were carried out by cold percolation. Dried, ground stembark (approximately 2.0 kg) was defatted in *n*-hexane at room temperature, followed by extraction three times with dichloromethane (10−15 L) over 3 days. Dichloromethane crude extracts of stembark (25.9 g) were obtained by evaporating the solvent in vacuo. Approximately 12 g of the stembark extract was fractionated over a High-Flash 5L column

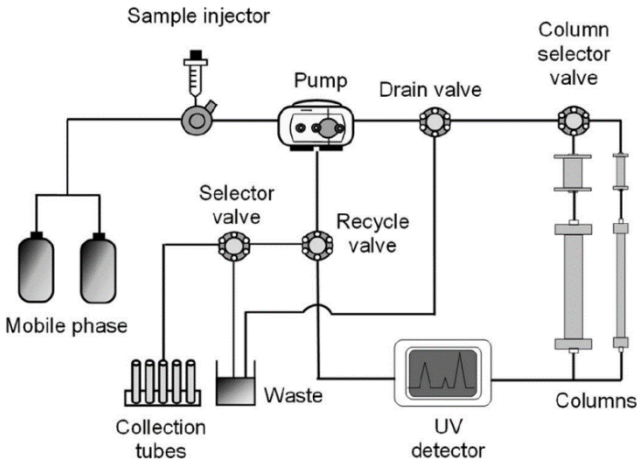

Figure 2. Schematic diagram of a recycling HPLC [15].

(60 x 180 mm) eluted with a gradient system of hexane-ethyl acetate (70:30 to 0:100) at a flow rate of 70 ml/min from 0 to 90 min to afford 16 fractions (F1 to F16) based on TLC analysis. Upon ^{1}H-NMR spectroscopy analysis, fraction F9 (1.2 g) and fraction F10 (5.2 g) were of interest whereby the spectra showed abundance of proton signals, hence provided a glimpse of compounds yet to be isolated and identified. Fraction F9 was re-fractionated over a Hi-Flash MPLC column, utilizing a gradient system of hexane-ethyl acetate (90:10 to 0:100) from 0 to 90 min, and yielded 13 subfractions (F9a-F9m). Fraction F10 was re-fractionated over a Hi-Flash MPLC column, with a gradient system of hexane-ethyl acetate (40:60 to 0:100) from 0 to 60 min, yielding 16 subfractions (F10a-F10p). Isolation procedures were done using semi-preparative HPLC. The unresolved peaks from semi-preparative HPLC were further purified using preparative recycling HPLC over a C18 column.

Preparative Recycling HPLC Conditions

The isolation and purification procedures were performed on a JAIGEL-ODS-AP preparative column (20 x 250 mm, 10 µm) eluted at 4 mL/min and detected at 215 nm. The maximum yield for each load was no more than 80 mg. Samples were dissolved in suitable solvent system, and partial-loop injections of 1-2 mL were performed on the injection loop. The recycle valve switches between recycling and eluting, thus enable the analytes to travel through the same column repetitively and improving separation in every cycle. While the system is still running, impurities can be removed by the drain valve, which channels the eluent to waste. The selector valve can be switched between collection and draining to collect the desired compound or drain the impurities. The column selector valve allows switching between two columns with different phases in recycling mode. Figure 2 shows a schematic diagram of the recycling HPLC system with its valve system [15].

RESULTS AND DISCUSSION

Most compounds from subfraction F9a-F9m were successfully afforded through semi-preparative HPLC [5,6], however, for subfraction F9j (58.6 mg), upon purification procedure using semi-preparative HPLC over ODS column (stepwise gradient solvent system of methanol-water, 70:30; flow rate 3.8 ml/min; UV detector 210 nm), the chromatogram showed a broad single peak and labelled subfraction F9j-i (9.6 mg) (Figure 3). Analysis of 1D and 2D NMR of the collected peak confirmed it as a mixture of two compounds. Subfraction F9j-i was then further purified using recycling HPLC over ODS column (isocratic solvent system methanol-water, 60:40; flow rate 4.0 ml/min; UV detector set at 215 nm). On the 7th cycle, it can be observed that the two major peaks were getting closer to each other and there was a possibility of both peaks to merge back into 1 peak. Therefore, on the 8th cycle within 190 min, the eluant was drained for the second peak, and when it was completely collected, the valve was then switched for the eluant to be channeled back to the column. After another two cycles of recycling, on the 11th cycle (310 min) another compound was collected. Both compounds were pure and named **1** (3.4 mg), a new bis-styryllactone while the neighboring peak was a styrylpyrone, **2** (1.5 mg) (Figure 4).

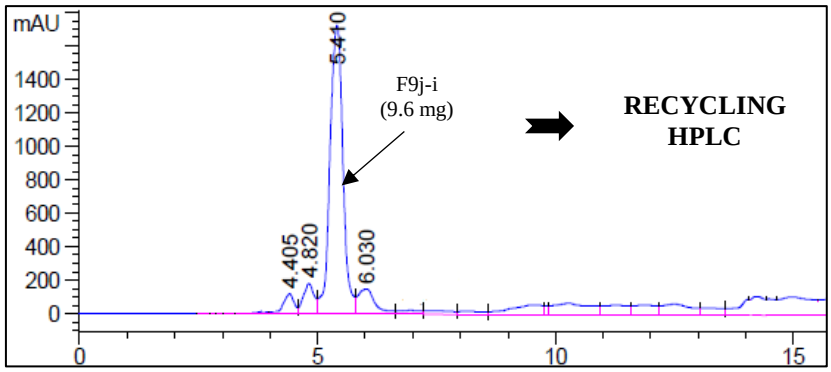

Figure 3. HPLC chromatogram for subfraction F9j

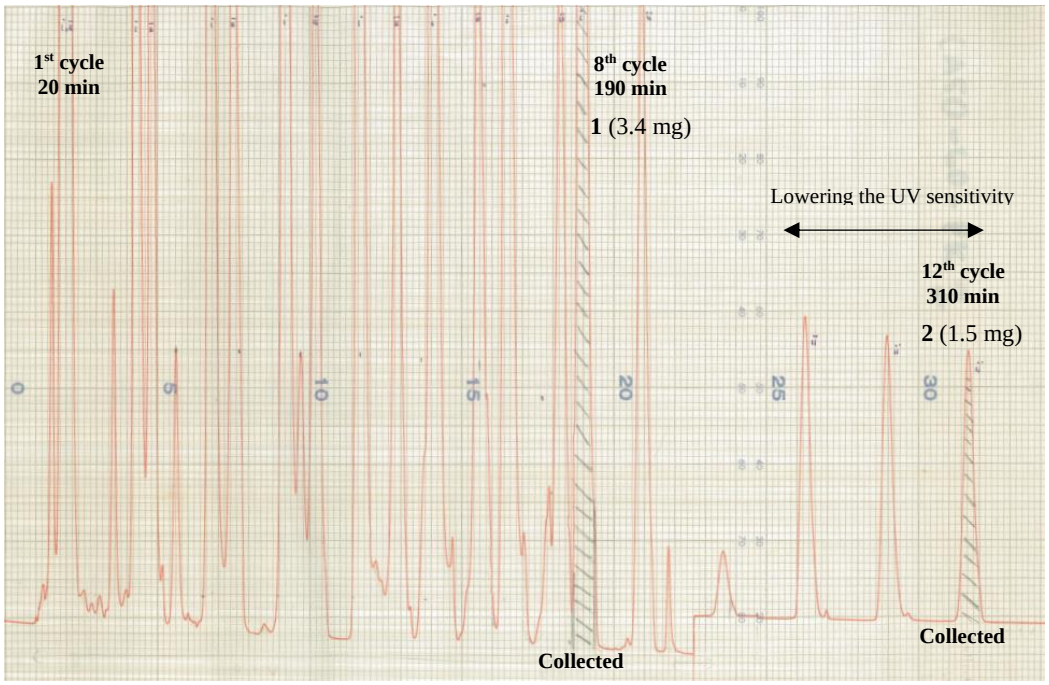

Figure 4. Separation of subfraction F9j-i using recycling HPLC

Most compounds from subfraction F10a-F10p were successfully afforded through semi-preparative HPLC, except for subfraction F10e, F10j and F10k. When subfraction F10e (279.5 mg) was purified using semi-preparative HPLC over ODS column (stepwise gradient solvent system of methanol-water; flow rate 3.8 ml/min; UV detector 210 nm), four peaks were observed (subfractions F10e-i, F10e-ii, F10e-iii, F10e-iv) (Figure 5). Besides subfraction F10e-ii, other subfractions were deemed pure compounds based on their ^{1}H-NMR analysis. Subfraction F10e-ii (6.9 mg) showed a 'shoulder' on the left side, indicating a mixture of compounds. It was further purified using recycling HPLC over ODS column (isocratic solvent system methanol-water, 50:50; flow rate 4.0 ml/min; UV detector set at 215 nm), and after a long cycle (20 cycles, 720 min), the peak was finally resolved into two peaks (Figure 6). Comparison of the ^{1}H-NMR spectra of each collected peaks with that of subfraction F10e-ii showed successful purification of two compounds (Figure 7). The isolates were identified as **3** (1.1 mg) and **4** (2.9 mg). Both compounds were found to have a similar molecular formula and were identified as isomeric pyranopyrone **3** and furanopyrone **4**.

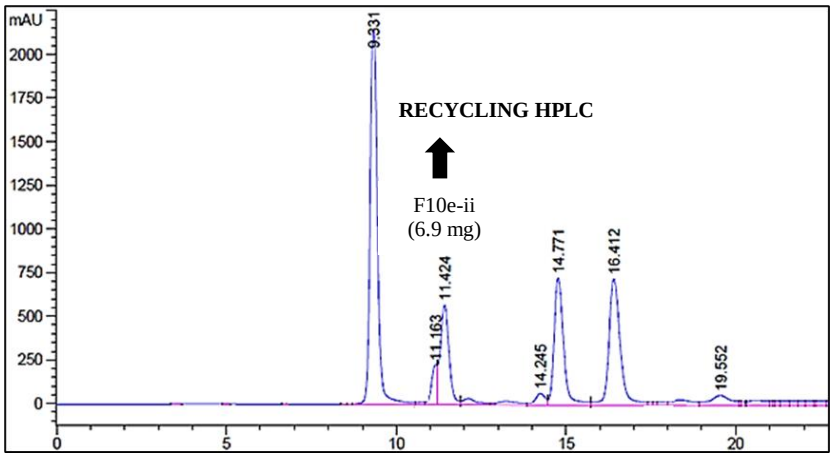

Figure 5. HPLC chromatogram for subfraction F10e

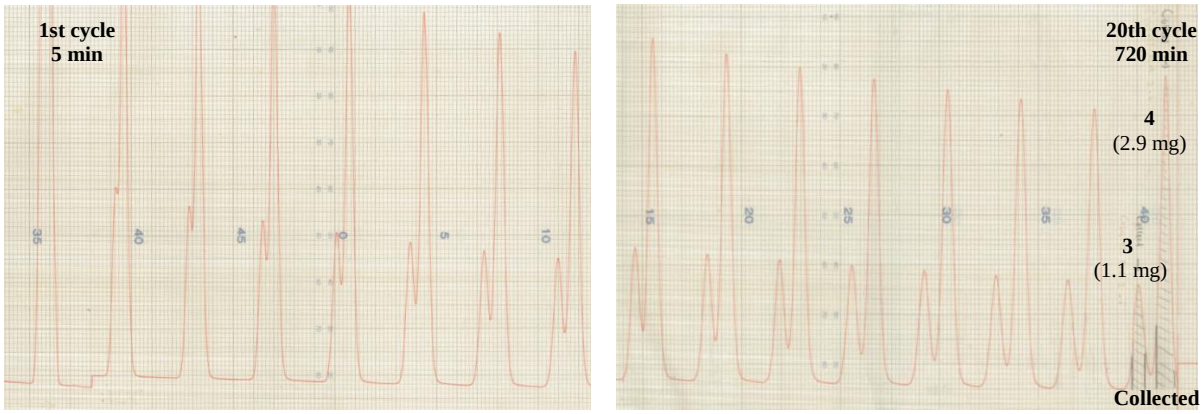

Figure 6. Separation of subfraction F10e-ii using recycling HPLC

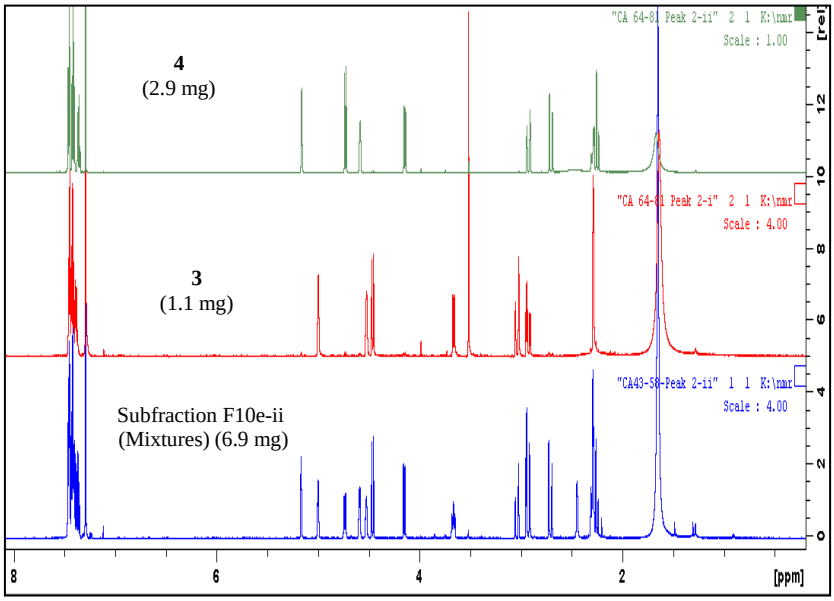

Figure 7. Comparison of ^{1}H-NMR spectrum of subfraction F10e-ii (mixtures) and its isolates, **3** and **4**

Subfraction F10j (156.0 mg) yielded a total of six peaks (F10j-i to F10j-vi) upon isolation by semi-preparative HPLC over ODS column using a stepwise gradient of methanol-water (flow rate at 3.8 ml/min, UV detector at 210 nm) (Figure 8). The major peak (F10j-iv), which occurred between 11 to 12 min with the gradient system methanol-water (70:30), was a mixture of compounds upon analysis of the ^{1}H-NMR spectrum, hence further purification was conducted. A yield of 31.8 mg (F10j-iv) was injected into a recycling HPLC over ODS column (isocratic solvent system methanol-water, 70:30; flow rate 4.0 ml/min; UV detector set at 215 nm). After the 1st cycle was completed, the valve was opened to drain out the impurities. Once the peak of the 2nd cycle started to show itself, the valve was then switched back to allow the eluant to be channeled back to the column, and this process was repeated until a straight-line baseline resolution (without impurities) was achieved. After 233 min, on the 9th cycle, two new bis-styryllactones **5** and **6** were successfully isolated (Figure 9). A comparison between the ^{1}H-NMR spectrum of the mixture (F10j-iv) with the ^{1}H-NMR spectra of both pure isolates is depicted in Figure 10.

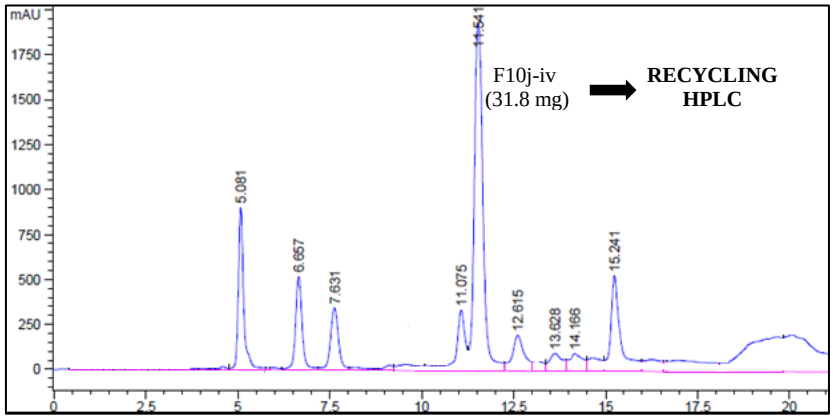

Figure 8. HPLC chromatogram for subfraction F10j

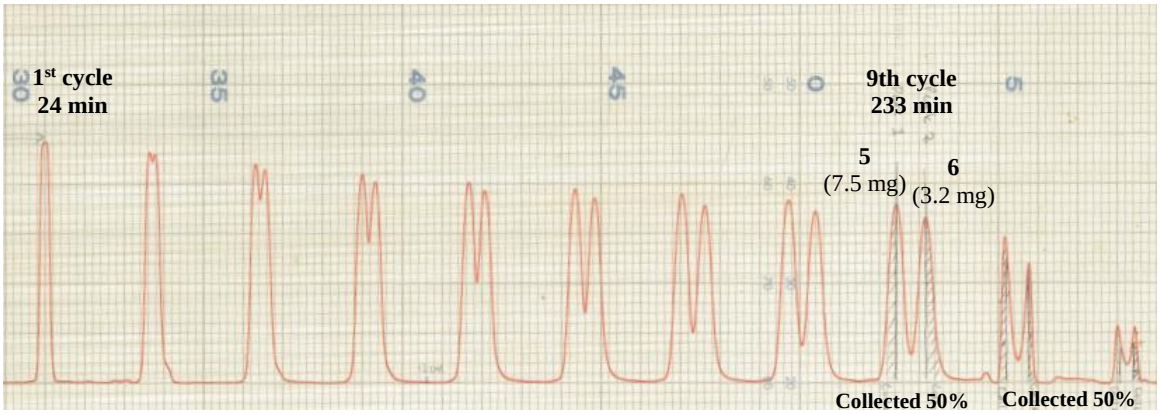

Figure 9. Separation of subfraction F10j-iv using recycling HPLC

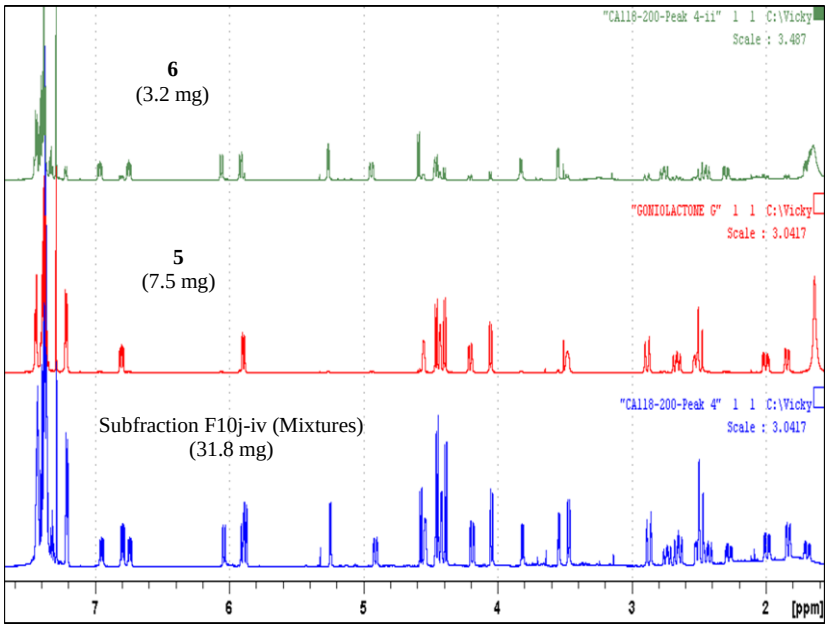

Figure 10. Comparison of ^{1}H-NMR spectrum of subfraction F10j-iv (mixtures) and its isolates, **5** and **6**

Subfraction F10k (89.4 mg) was isolated using semi-preparative HPLC over ODS column with a stepwise gradient system of methanol-water (flow rate 3.8 ml/min; UV detector 210 nm) to afford subfractions F10k-i, F10k-ii and F10k-iii (Figure 11). The last peak appeared at a retention time of 9.9 min, gradient system methanol-water (80:20), and upon checking for purity using ^{1}H-NMR experiment, subfraction F10k-iii (14.6 mg) was still in need of further purification, therefore recycling HPLC over ODS column (isocratic solvent system methanol-water, 70:30; flow rate 4.0 ml/min; UV detector set at 215 nm) was utilized to purify this subfraction. After the 1st cycle was completed, the valve was opened to drain out the impurities seen on the baseline. Therefore, after the 2nd cycle, the impurities were lesser, and the focus was prioritized to the three peaks. After eight cycles in a period of 240 min, a pure isolate, **7** (5.7 mg), a new bis-styryllactone was collected (Figure 12). The yield of the two neighboring peaks was too low to be detected using ^{1}H-NMR. A comparison between the ^{1}H-NMR spectra of the mixture (F10k-iii) with the purified compound **7** is depicted in Figure 13.

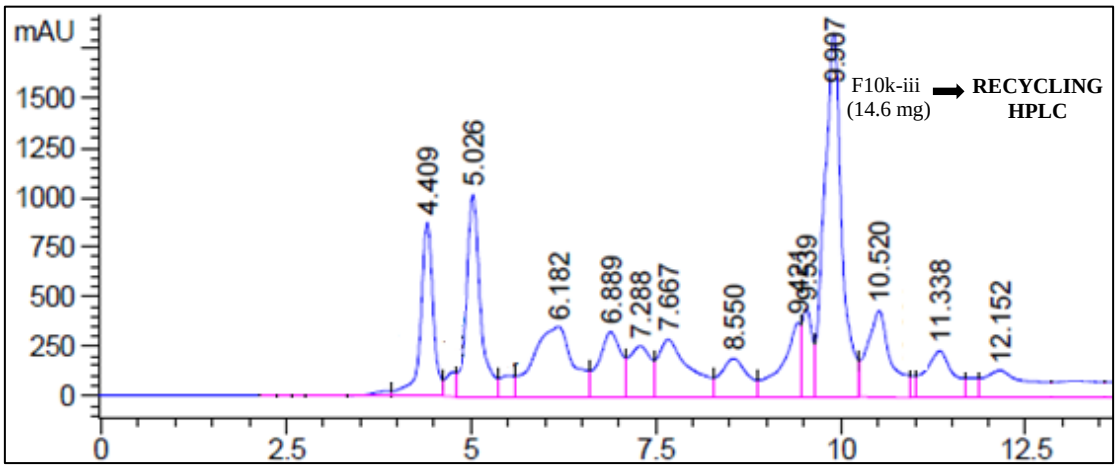

Figure 11. HPLC chromatogram for subfraction F10k

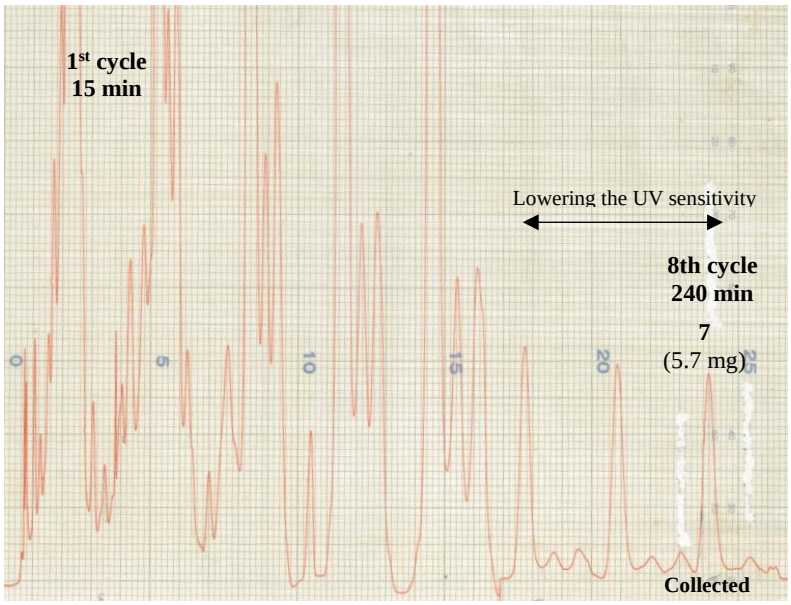

Figure 12. Separation of subfraction F10k-iii using recycling HPLC

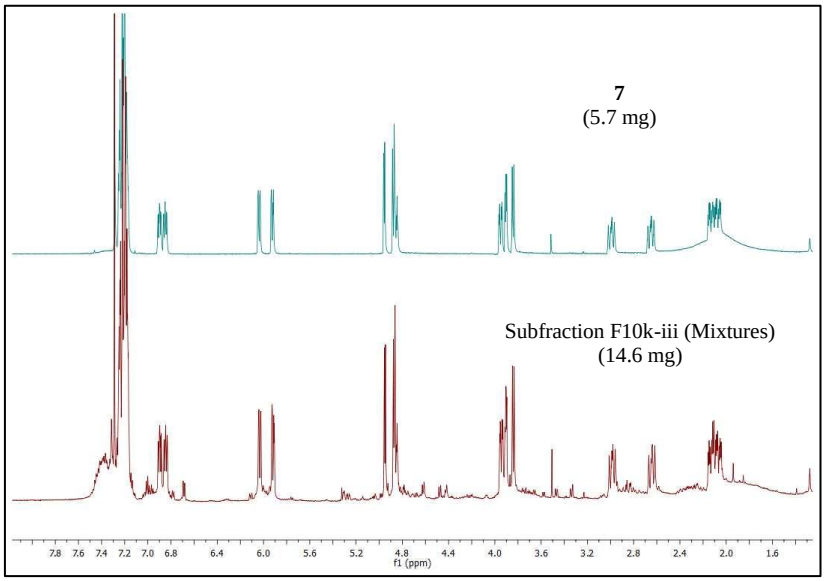

Figure 13. Comparison of ^{1}H-NMR spectrum of subfraction F10k-iii (mixtures) and its isolate, **7**

Preparative recycling HPLC is a very effective method for isolating of complex mixtures and purifying of compounds using diverse stationary phases. In recycling HPLC, the unresolved peaks are recycled back to the column through a recycle valve in the HPLC system. This increases the separation efficiency and minimizes peak dispersion. Furthermore, unlike conventional HPLC, fresh solvent is not required during the recycling process, which is another advantage to this instrument. Recycling HPLC needs to be popularized among natural product chemists to overcome the tedious process of conventional phytochemical isolation, which often leads to re-isolation of common metabolites and is a costly pitfall in isolating natural products and a waste of time and human resources.

Identification of Compounds

The structural elucidation of bis-styryllactones **1**, **5**, **6** and **7** was thoroughly discussed in previously published paper by our group, Bihud et al. (2019) [5], while styryllactones **2**, **3** and **4** were reported by Rasol et al. (2018) [6].

CONCLUSION

In this study, seven minor compounds with a very low yield were successfully isolated and purified using preparative recycling HPLC. Four of the compounds were new bis-styryllactones, goniolanceolatin B, goniolanceolatin C, goniolanceolatin F and goniolanceolatin H, which represent a rare naturally occurring dimer, incorporating two monomers, either a pyranopyrone unit and a styrylpyrone unit, or two styrylpyrone units, which are linked by an ether-bridge at positions C-8/C-8', C-8/C-7', or C-7/C-8'. Another three compounds were styryllactones, (6*S*,7*S*, 8*S*)-goniodiol-7-monoacetate, (1*S*,5*S*,7*R*,8*S*)-3-exo,7-endo-(+)-8-epi-9-deoxygoniopypyrone and (1*S*,5*S*,7*S*, 8*S*)-(-)-goniofupyrone B. Recycling HPLC gave high recovery rate with excellent purity, thus minimizing the amount of sample wastage. This approach expediate the isolation and purification of compounds, while minimizing the cost and time of the research.

ACKNOWLEDGEMENTS

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