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Research Article

Mass Fragmentation Patterns as Fingerprints in Identification of Known Oligostilbenes in *Dryobalanops* Spp. Extracts

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ABSTRACT
An ultra-performance liquid chromatography (UPLC) coupled with electrospray ionization (ESI) tandem mass spectrometry was establish to dereplicate oligostilbenes in three Dipterocarpaceous plant extracts; *Dryobalanops rappa*, *D. aromatica* and *D. lanceolata*. Two compounds, hopeaphenol (**1**) and vaticaphenol A (**2**) were identified by comparing their mass spectrometric data with an in-house database. Unidentified compounds were isolated and purified using automated chromatographic techniques, including analytical and preparative HPLC. The compounds were characterized by means of spectroscopic analyses, including ultra-violet (UV), nuclear magnetic resonance (NMR) and mass spectrometry (MS). The compounds were characterized as malaysianols A (**3**) and B (**4**), vaticanols B (**5**) and C (**6**), ampelopsin E (**7**), a-viniferin (**8**), e-viniferin (**9**), and nepalensinol B (**10**). The compounds are known to science, though new to our collection, thus not listed in our database. Hence they are unidentifiable in dereplication process prior to their isolation.

Keywords: *Dryobalanops*, Oligostilbene, Dereplication, Chromatography, Spectroscopy.

INTRODUCTION

The identification of chemical compounds from plant extracts in natural products research often involves tedious, time-consuming and expensive process. Selecting the strategy for the compound identification is essential with respect to the time and efforts spent on discovery of active natural compounds. Conventional natural products discovery programs rely on the fractionation, isolation and purification of crude extracts as well as structural elucidation of pure compounds[1-3]. This tedious, time consuming and expensive process may end up with disappointing outputs when isolating well known previously characterized compounds. Dipterocarpaceae is a relatively small family of trees with about 270 members found in Malaysian dipterocarp forest. The Dipterocarpaceae family includes only trees, which vary from medium to large sized, usually evergreen and rarely deciduous in the dry season. Dipterocarps are distributed from sea level to about 1200 metres. The trees are dominant in tropical forest. *Dryobalanops* is a unique genus of dipterocarpaceous plant, as it only consists of seven species worldwide. Most of them are found in tropical forest of Peninsula Malaysia, Sumatera, Indonesia and Borneo. Like other genera from Dipterocarpaceae family, *Dyrobalanops* also rich in stilbene oligomers. In recent years, stilbenes, which exist in natural kingdom, have attracted much attention for their various biological activities, including antimicrobial, anti-cancer, anti-inflammation, hepatoprotective and hepatotoxic activities[3]. Plants from the Dipterocarpaceae family have proven to be a rich source of oligostilbene compounds derived from a stilbene, resveratrol (4,3,5'-trihydroxystilbene). To date, about 100 out of more than 400 stilbene derivatives reported were isolated from Dipterocarpacae plants[4]. With the rising number of characterized compounds, the possibility of re-isolating known compounds from natural resources has increased. The complexity of naturally occurring compounds requires high-end spectrometric techniques, highly skilled operator and time-consuming elucidation works. This study presents the characterization of compounds in crude plant extracts by a dereplication process using LC-MS^n. The characterized compounds were hopeaphenol and vaticaphenol A, oligostilbenes extracted from dipterocarpaceous plant *Dryobalanops rappa*, *D. aromatica* and *D. lanceolata*. High performance liquid chromatography (HPLC) separation of the extract led to isolation of eight resveratrol oligomers.

MATERIALS AND METHODS

General experimental procedures

Mass spectra were obtained on an Agilent ion-trap mass spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, HMQC, HMBC and NOESY experiments, were recorded

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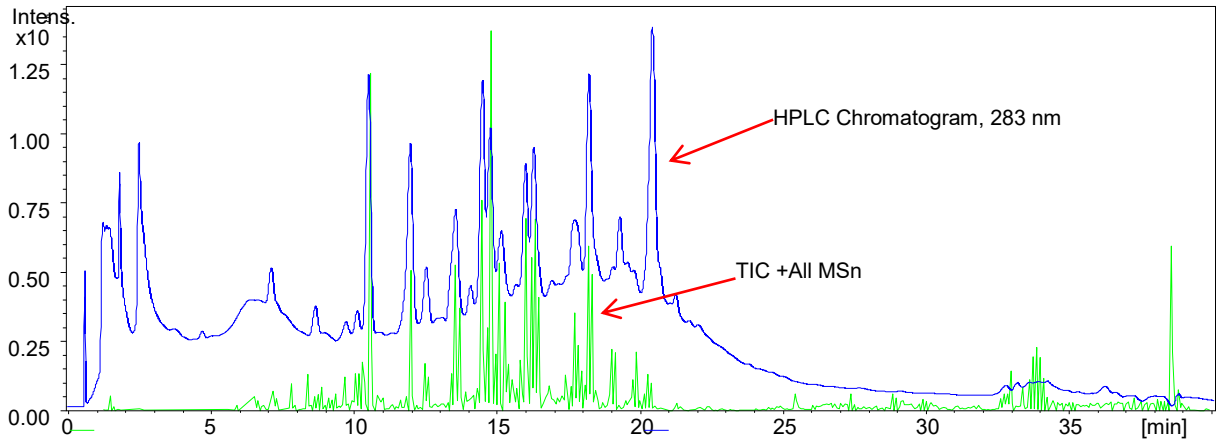


Figure 1: Chromatographic profile of HPLC and MS analyses

on a BRUKER AVANCE 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) with chemical shifts given in ppm (δ) and coupling constant (J) were given in Hz. HPLC analyses were conducted using an Agilent 1120 with DAD detector. Hypersil ODS C18 column (5µ; 150 × 4.6 mm ID) was used for analytical and Supelco (150 × 20.0 mm ID) for semi-preparative HPLC.

Plant materials

A classical extraction technique, which is maceration, was used for the extraction of the plants. The technique is based on the extracting power of different solvents in use and the application of mixing. The plant material was ground into small particle. In maceration process, a mixture of water and acetone (1:1) was used and the sample was left macerated overnight. Later the extract was strained off and the extraction solvent was removed to obtain a crude residue. The process was repeated until the test for phenolic presence was negative.

Optimization of chromatographic conditions

The chromatographic conditions were obtained on an analytical HPLC system. This includes choice of the chromatographic column, temperature, solvent system, gradient profile and flow rate of delivery solvent. The mobile phase consisting of 0.1% TFA in water (A) and acetonitrile (B) was run with gradient elution at a flow rate of 1.0 mL/min. The linear gradient elution was set as follows: 0 □ 40 min; 10% B → 60% B. The injection volume was 10 □L. UV absorption was monitored at 215 and 283 nm. The column temperature was maintained at 30°C. The optimized chromatographic conditions were used in the preparative HPLC for isolation and purification purposes. Apart from the chromatographic column, very fraction has a specific chromatographic condition.

Mass spectrometric condition

The mass data were obtained on an ion-trap system. The liquid chromatographic system followed the analytical HPLC system, with different flow rate, which was 0.7 ml/min, to suit the MS system requirement. The injection volume was 2.0 □l, capillary voltage 3.3 kV, source temperature 120°C and collision gas 0.14ml/min.

Isolation and purification of oligostilbenes

The crude drug was injected into preparative HPLC using individually optimized chromatographic conditions. Each sample was prepared as 450 mg in 10 ml methanol. The separation was carried out over a Supelco (20 x 150mm), 5 µm semi preparative column. The detection was at wavelength 215nm and 283nm. The process was run repetitively until enough samples were obtained for further purification. The purification procedure was similar as of the isolation, but using smaller column ID. The process was repeated enough until only one peak observed in the chromatogram indicating pure compound.

Compound identification

The purified sample was dried using rotary evaporator. The sample was dissolved in deuterated acetone and transferred into a NMR tube. The NMR data were recorded for each sample.

RESULTS AND DISCUSSION

Dereplication strategy for compound identification

For the rapid identification of the oligostilbenes, the methanolic crude extract was directly injected into the ion trap LC-MS system. A 1-mg/ml sample was prepared in acetonitrile and was analyzed in different experiments. The chromatographic profile of Total Ion Chromatogram (TIC) of the crude extract was obtained. The retention times were different with those of HPLC analysis (Figure 1). Both analyses were performed under different chromatographic conditions, where the column and flow rate were adjusted to suit the instrument requirements and for better resolution. Altogether, the retention times were different but the sequence of elution seemed to be identical. The intensity of the peaks in both chromatograms is also different. This can be explained by considering the difference in the detection method for both UV and MS techniques. The UV detector measures the molar absorptivity whereas the MS detector measures the ionizability of the compounds. The response might be quite different for the same compound when detected by UV or MS.

The TIC of the LC-MS analysis provides on-line molecular mass information. This helps in estimating the oligomerization degrees of the stilbenes. As the resveratrol (the biogenetic precursor of all oligostilbenes isolated in this work) mass is 228, a dimeric stilbene mass would be around 450-480, considering inter-monomer bonding and the possibility of excess ogygen atoms. A trimeric stilbene mass would be around 680-700 and for a tetrameric

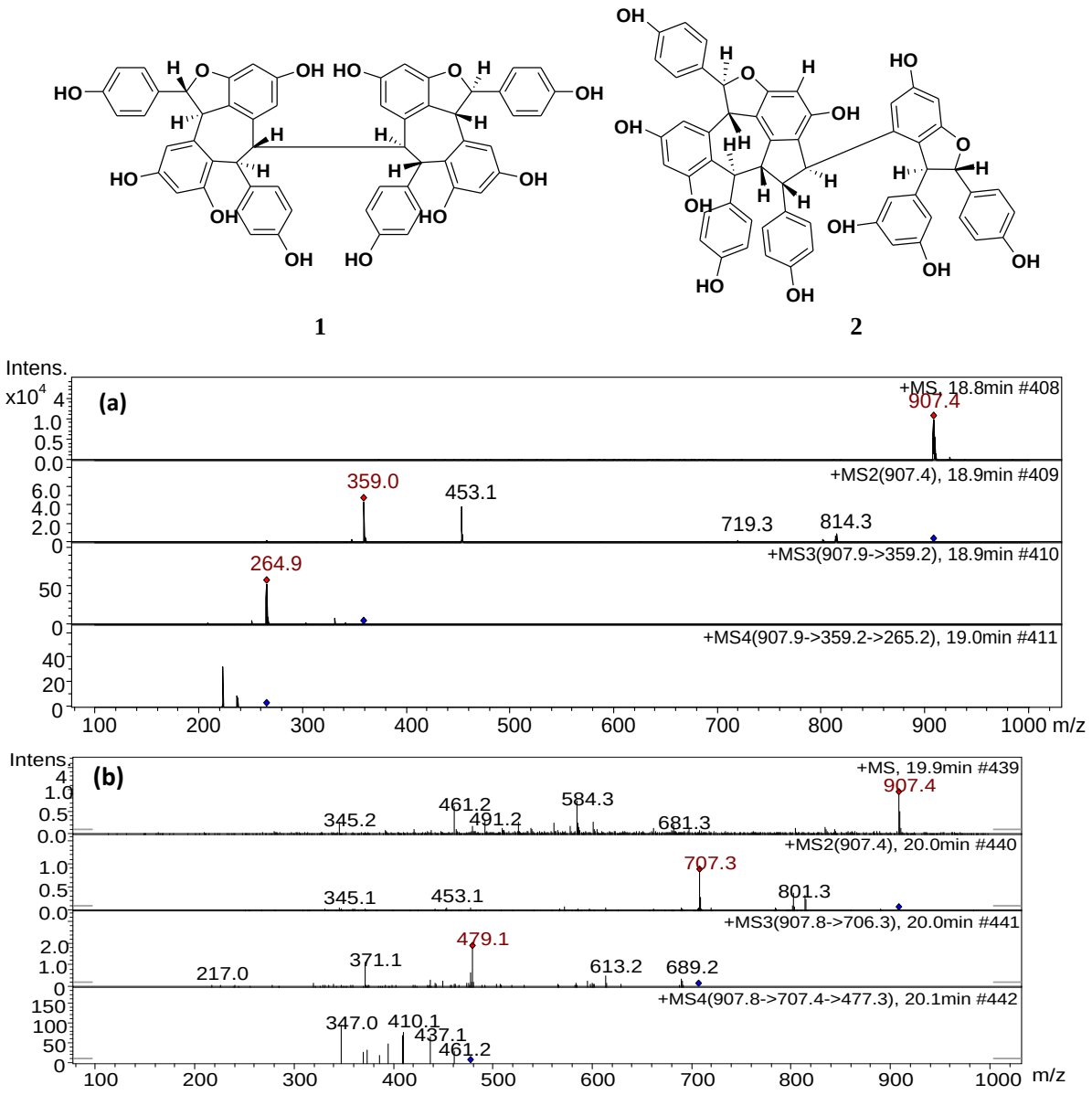


Figure 2: Mass fragmentation patterns of MS, MS^2, MS^3 and MS^4 for **(a)** hopeaphenol and **(b)** vaticaphenol A.

stilbene, around 900-950.

The analyses were continued with MS^2, MS^3 and so on until there are no more ions to be fragmented. The fragmentation patterns extracted from the experiments were compared with those of the pure compounds isolated previously. The fragmentation patterns are unique for each compounds regardless the retention times and intensity of their peak. This will ensure positive identification directly from a mixture. After considering the co-elutions and overlapping peaks, only two compounds were positively identified, which are hopeaphenol and vaticaphenol A. Figure 2 shows fragmentation patterns of hopeaphenol (**1**) and vaticaphenol A (**2**) from the crude extract of *Dryobalanops lanceolate*, *D. rappa* and *D. aromatica*, which are identical with those in the library.

Upon identification of known compounds from their mass fragmentation patterns, the chromatographic peaks correspond to the compounds are distinguished. It is important to distinguish the peaks that represent known compounds in dereplication process so that the same compound would not be further separated and isolated. Figure 3 shows the chromatogram of the crude extract and the identified peaks correspond to the compounds from the library.

Isolation and purification of unidentified compounds

The chromatograms of crude extracts from the *Dryobalanops* shown in figure 3 were used as references for further isolation of compounds. Those compounds, which were identified in the dereplication process, were not isolated. This will safe time, energy and resources as only unknown compounds were underwent the isolation procedures. Isolation of compounds from *D. lanceolata* leads to 6 different fractions, *D. rappa* (6 fractions) and *D. aromatic* (3 fractions). All fractions underwent further HPLC analyses for compounds separation. Similar

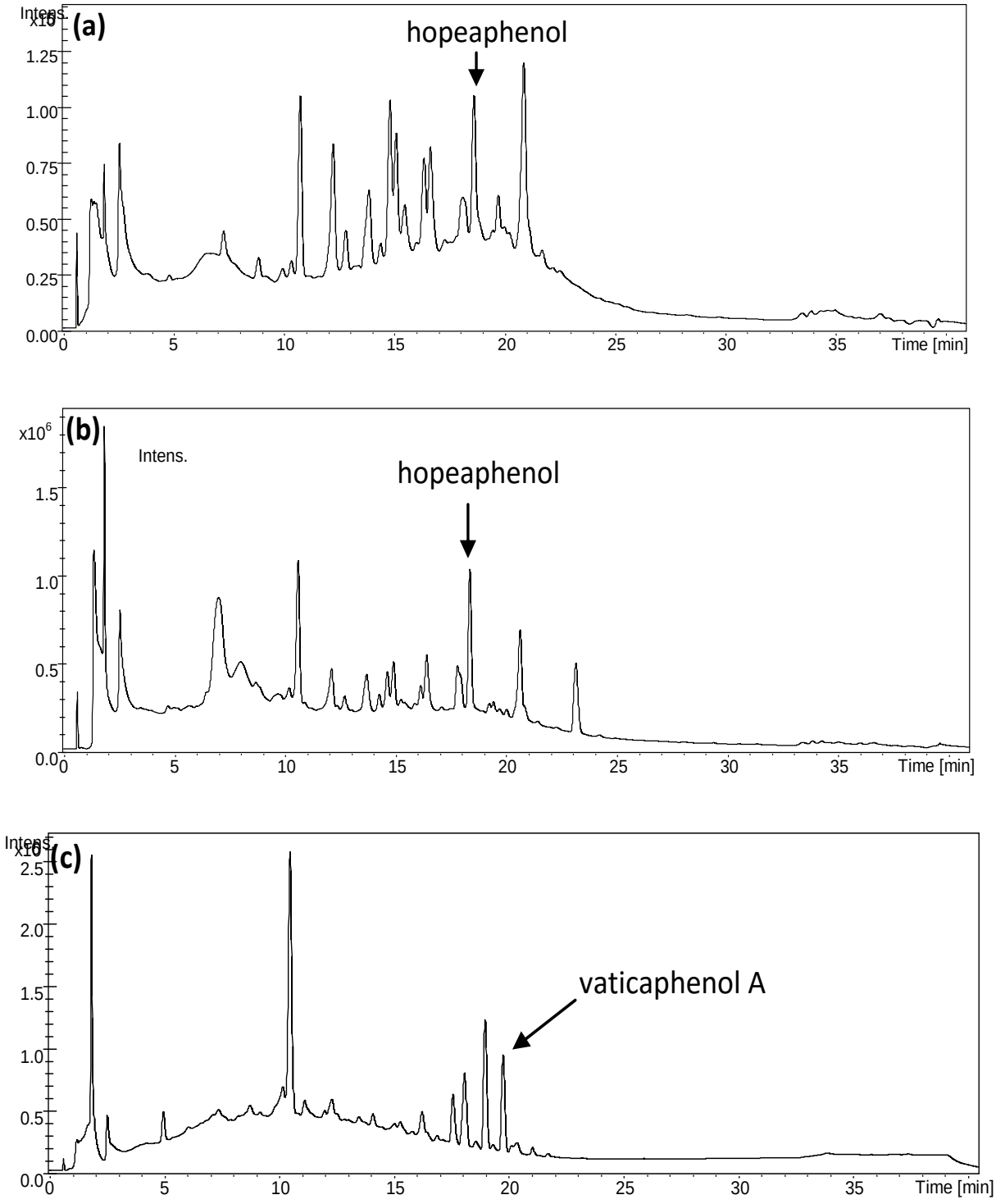


Figure 3: Identification of compounds from the crude extracts of **(a)** *Dryobalanops lanceolata*; **(b)** *Dryobalanops rappa*; and **(c)** *Dryobalanops aromatica*.

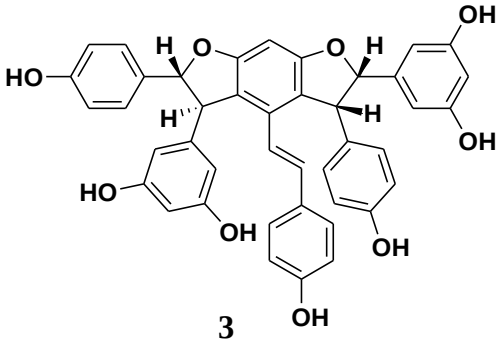
compounds were combined and those impure were purified.

Identification of isolated compounds

Since all isolated compounds are known, the identification was done by comparing the ^{1}H-NMR with the reported data[5-9]. Eight compounds were successfully identified due to their spectroscopic analyses and the others were insufficient for identification. The identified compounds are as follow: malaysianols A (**3**) and B (**4**), vaticanols B (**5**) and C (**6**), ampelopsin E (**7**), a-viniferin (**8**), e-viniferin (**9**), and nepalensinol B (**10**).

CONCLUSION

The analysis of the isolated oligostilbenes with LC-MS ion trap system showed the competency of the system to distinguish oligostilbene directly from a crude extract. The



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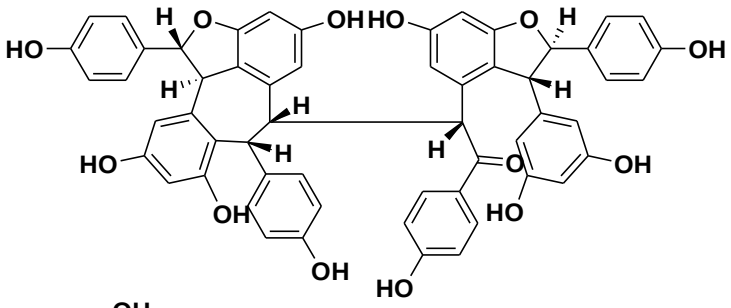

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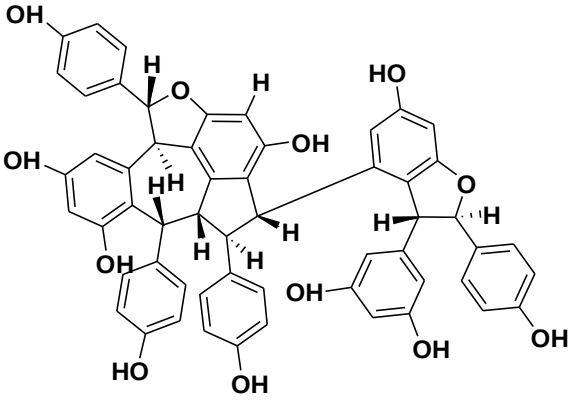

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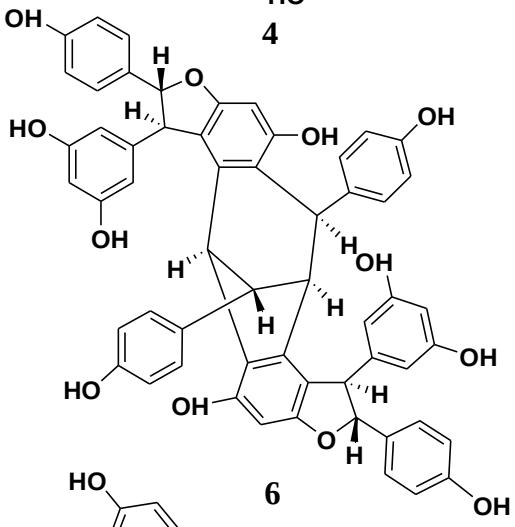

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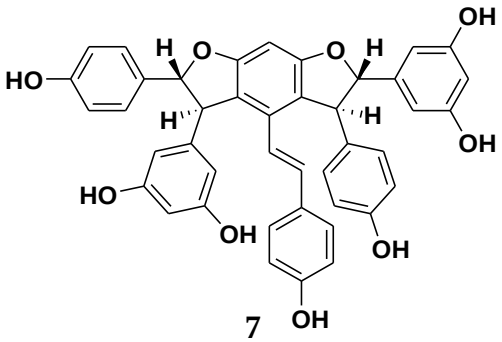

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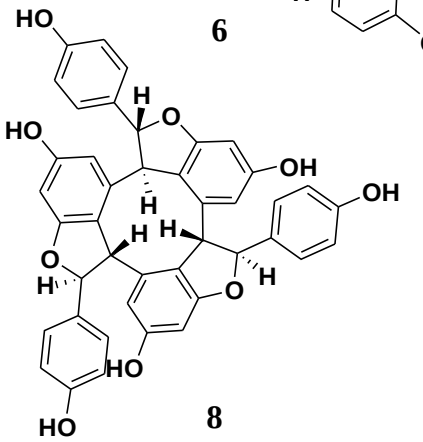

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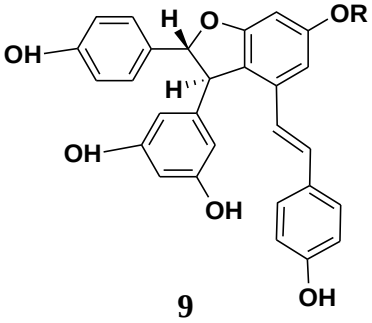

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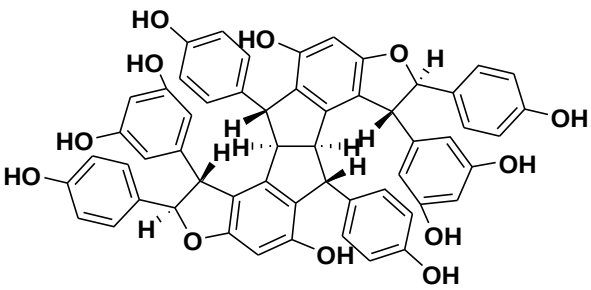

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system was recognized as being able to successfully identify a known compound solely from its fragmentation pattern, regardless of the retention time, or other data. This allows to eliminate the dependence on the chromatographic conditions and selection of column.

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