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Research Article

A SELECTIVE CHEMICAL METHOD FOR THE SEPARATION OF QUINONES FROM THE STEM BARK OF *DIOSPYROS ANISANDRA*

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ABSTRACT

Stem bark extract from *Diospyros anisandra* has been shown to possess significant antimycobacterial activity against a resistant strain of *M. tuberculosis*. This activity was detected in the quinone-rich fractions of the non polar extract. The bioassay-guided fractionation of the non-polar extract, resulted in a considerable amount of known terpenoids, and fatty esters, making it difficult to isolate the active quinoid components. This article deals with the comparison of four methods of extraction in combination with KOH/HCl treatment for the separation of slightly polar quinones from the terpenoids and fatty ester mixture. Ground plant material was extracted with *n*-hexane by static maceration (24 h), dynamic maceration (24 h), Soxhlet extraction (4 h) and ultrasonic extraction (2 h). The extraction efficiencies were measured and then, each extract was dissolved in chloroform and partitioned with an aqueous solution of KOH 5%; the aqueous solution was washed with chloroform, acidified until pH 5 with a chloride acid solution and extracted again with chloroform. The comparison of each fraction obtained was carried out by TLC and CG-MS analysis. Quantification of each quinone component by GC-MS revealed that plumbagin and droserone were the major naphthoquinones in the hexanic extract of the steam bark of this species. The four methods employed in combination with KOH/HCl allowed the separation of slightly polar quinones from terpenoids and fatty ester mixtures from the stem bark extract of *D.anisandra*.

Keywords: Quinone, KOH/HCl extraction, Plumbagin, Maritinone, Droserone, *Diospyros anisandra*

INTRODUCTION

Quinones are a commom type of secondary metabolites in bacteria and plants that often exhibit interesting biological activities. These compounds are involved in defense mechanisms by plants against predators. Among the most important biological activities reported on quinones are cytotoxic [1], antioxidant [2], anti-inflammatory [3], antileishmanial [4], antimalarial, antimycobacterial, antifungal [5] and antitermitic activities [6]. Some of the genus rich in quinone components is Diospyros, from about 500 species, widely distributed in tropic and subtropics regions [7]. As part of our phytochemical and pharmacological evaluation on Diospyros species, we have described previously the antimycobacterial evaluation of *Diospyros anisandra*, a plant endemic to Yucatan, Mexico where is used in traditional Mayan medicine to treat skin infections [8,9]. Quinone-rich fractions from this plant has shown to possess interesting antimycobacterial activities [8]. Quinones are more likely to be lipid-soluble and co-extract from crude plant extracts with carotenoids and chlorophylls among other lipid-soluble components [10]. During the bioassay-guided fractionation of the non-polar extract of *D. anisandra*, a considerable amount of known terpenoids and fatty esters, which were inactive against *M. tuberculosis* resistant strains, was also obtained, making it difficult to isolate the active components [11].

Some of the classical techniques reported for extracting slightly polar quinones involve maceration, ultrasonic waves extraction and Soxhlet extraction as reported by De Paiva et al [12] and Grevenstuk et al [13] on the extraction of plumbagin. The formers determined that Soxhlet extraction with chloroform was the most efficient method for plumbagin extraction; however, prolonged heating time in the extraction promoted plumbagin degradation. Furthermore, palmitic acid and β-sitosterol were obtained in addition to quinone. The latter compare Soxhlet and ultrasound-assisted extraction methods on plumbagin from *Drosophyllum lusitanicum* and found no significant differences between both techniques. Furthermore *n*-hexane proved to be the most suitable solvent for extracting plumbagin. Nevertheless, this method also extracted droserone, neophytadiene and palmitic acid. These previous works show the lack of selectivity in obtaining quinone-rich fraction.

Quinones easily undergo addition and substitution reactions to form a semiquinone radical that can be oxidised back to the quinone form by an electronic transference reaction. Reversible reduction to a colourless form and restoration of the colour by aerial oxidation is characteristic of quinones. This principle is used to identify quinones, which turn red and purple in the presence of basic solutions [10]. During these reactions, the solubility properties of slightly polar quinones changes and it is possible to dissolved these molecules in polar solvents as aqueous solutions. Taking into account this characteristic and the lack of selective and efficient methods for obtaining quinones, the objective of the current research was to compare various methods to extract quinones from the stem bark of *Diospyros anisandra* including static maceration, dynamic maceration, soxhlet extraction and ultrasound-assisted extraction in combination with KOH/HCl treatment. Furthermore, the concentration of each quinone and the yield for each extraction method were compared.

MATERIALS AND METHODS

Materials and reagents

Stem bark from *D.anisandra* was collected from Libre Union-Yaxcaba, Yucatán in October 2007. The plant material was identified and a voucher specimen of the sample (Collection number 1474) was preserved in the herbarium of Centro de Investigación Científica de Yucatán (CICY). Chloroform and *n*-hexane were of analytical grade and purchased from Fermon (Nuevo Leon, Mexico). All naphthoquinone and terpenoids references were isolated from the stem bark of *D. anisandra* and identified previously by spectrometric techniques [11]. Silica gel 60 F_{254} aluminium plates (Merck 0.2 mm thickness) were used for analytical TLC. The components were visualised under UV/Vis light at 254 and 365 nm in a UV cabinet and by using a solution of phosphomolybdic acid (20 g) and ceric sulphate (2.5 g) in 500 ml of sulphuric acid (5%), followed by heating. Quinones were detected by spraying the plate with a solution of 5% KOH in ethanol followed by heating for 5 minutes at 105°C.

Sample preparation and extraction procedures

Stem bark of *D. anisandra* were air-dried and ground on a Brabender Dusiburg mill using a No 2 Sieve. Powdered stem bark (20 g) was extracted with 250 mL of *n*-hexane by four different extraction techniques: static maceration (24 h), dynamic maceration (24 h), Soxhlet extraction (4 h) and ultrasonic extraction (2 h). Each extraction technique was repeated three times. The extracts obtained were evaporated to dryness under reduced pressure and injected into a gas chromatograph coupled to a mass spectrometer (GC-MS). After GC-MS analysis, each extract was dissolved in 500 mL

of chloroform and partitioned with an aqueous solution of KOH 5% (250 mL); the aqueous solution was washed with chloroform, acidified with a chloride acid solution 10% (300 mL) until pH 5 and extracted again with chloroform. All fractions were evaporated and the extraction efficiency was calculated.

GC-MS analysis

A sample of each extract and pure compounds obtained were dissolved in chloroform (1 mg/ml) and were analysed on an Agilent Technologies 6890 N gas chromatograph interfaced to an Agilent Technologies 5975B inert mass selective detector (MDS). The chromatographic fractionation was repeated three times to guarantee the reproducibility of the experiment. The analysis was performed on an Ultra 1 capillary column (25 m x 0.321 mm i.d.; 0.52 µm df; 100% dimethylpolysiloxane). Helium was used as the carrier gas at a constant pressure (1.5 mL/min). The initial oven temperature was kept at 180°C for 5 min and then ramped up to 300°C at 10°C/min. Spectroscopic quantification of all naphthoquinones in each extract were performed in a Hewlett Packard 5890 gas chromatograph using an Ultra 1 capillary column (25 m x 0.321 mm i.d.; 0.52 i.d.; 0.52 µm df; 100% dimethylpolysiloxane). Nitrogen was used as a carrier gas under constant pressure (1 ml/min). The initial oven temperature was kept at 180°C for 5 min, and ramped up to 300°C at 10°C/min in 20 min. External standard methodology described by Pavia et al. [13] was used. All calibration solutions were prepared in chloroform at concentrations ranging from 0.1 to 7 µg/µL.

Statistical analysis

The data were analysed by ANOVA to assess if there were any significant differences between the extraction methods and the total content of each naphthoquinone recovered by the different extraction methods. All experiments with significant differences were compared using Tukey post-hoc test ($p = 0.05$). All calculations were performed with GraphPad Prism 5 statistical package.

RESULTS AND DISCUSSION

The extraction efficiencies of the stem bark of *D. anisandra* were measured for four extraction methods: static maceration, dynamic maceration, Soxhlet and ultrasonic extraction to determine which method resulted in the highest amounts of the hexanic extract for further purification. In this study hexane was selected as a solvent system based on observations made by Grevenstuk et al. [13] on the extraction of plumbagin. Soxhlet extraction was the most efficient technique and resulted in high amounts of extract (Figure 1). Extraction with the Soxhlet apparatus for 4 hours resulted in ten times more extract than the other three techniques. The efficiency of Soxhlet extraction is due to the continuous renewed of the solvent, promoting more interaction between the plant material and the solvent. However, it also extracted the higher amounts of terpenoids and fatty esters together with quinoid components. No significant differences in efficiency were observed between dynamic maceration and ultrasonic extraction, likely because both methods use physical forces to transfer all of the cellular components to the solvent medium. However, ultrasonic extraction with hexane required only two hours whereas extraction by dynamic and static maceration took 24 hrs. Although static maceration gave the lowest amount of hexanic extracts it also gave the lowest amount of terpenoids and fatty esters in comparison with other methods, meaning that the extraction of a compound is determined by its affinity to the hexanic solvent [13].

There were no qualitative differences in the chemical composition between the different extraction methods by CG-MS. TLC analysis of each extract also gave similar profile of compounds in all the extraction methods, and demonstrated the presence of eight main compounds. The results indicate the inability to obtain a quinone fraction free of terpenoids and fatty esters. It is noteworthy that the separation of this complex mixture of quinones, terpenoids and fatty esters is a time-consuming task. The purification method consists of repetitive chromatography on silica gel columns and gel permeation chromatography using different solvent systems based primarily on hexane, dichloromethane, ethyl acetate and methanol as eluents. However, in most of the cases, fractions found to be pure by TLC were determined to be mixtures by GC-MS. Treating each extract with KOH and HCl resulted in a quinone-rich fraction that was further quantify by GC-MS. TLC plate analysis (Figure 2) of the hexanic extract obtained by static maceration without treatment (E) and the quinone (Q) and terpenoid (T) fractions after KOH/HCl treatment together with GC-MS of the quinone and terpenoid/fatty ester fractions (Figure 3 and 4) also corroborate the efficiency of the method employed. The following compounds were identified in the quinone fraction: plumbagin (**1**), maritinone (**2**), 3'3-biplumbagin (**3**), and droserone (**4**) (Figures 4, 5 and 6). The terpenoid/fatty ester fraction was constituted by lupeol, betulin, betulinic acid, octadecenoic acid methyl ester, hexadecanoic acid methyl ester, 9,12-octadecadienoic acid methyl ester, and a naphthalenone type compound [10] (Figures 3, 5 and 6).

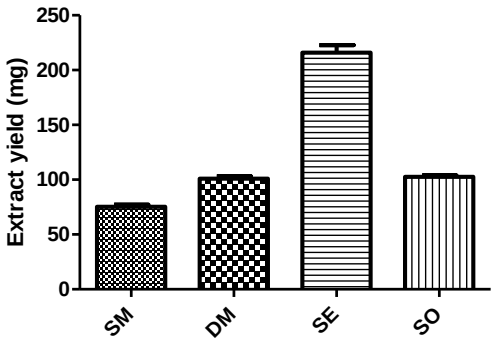

Fig. 1: Yields (%) of the *n*-hexanic extracts from the stem bark of *Diospyros anisandra* obtained by static maceration (SM), dynamic maceration (DM), Soxhlet extraction (SE), and ultrasound-assisted extraction (SO).

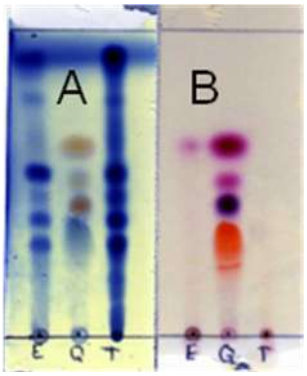

Fig. 2: Thin-layer chromatography of the *n*-hexanic extract obtained by static maceration without treatment (E), and after treatment with KOH/HCl. (Q): quinone-rich fraction, (T): terpene-rich fraction. TLC plate A was reveled with phosphomolibdic acid and TLC plate B was revealed with 5% KOH in ethanol. Solvent system: *n*-hexane:acetone (8:2).

Figure 7 shows the type of quinones obtained from each extraction method and the concentration of each quinone for 10 mg of total extract. Droserone is the major quinone found, followed by plumbagin, 3,3-biplumbagin and maritinone. The proportion of each quinone was similar in all the methods tested. However, with regard to the recovery of plumbagin and droserone, static maceration at ambient temperatures resulted in the higher amounts of both quinones compared to Soxhlet extraction. The low recovery of plumbagin and droserone by Soxhlet extraction could be due to the high volatility of these quinones in a medium that is heated during extraction[14]. In fact, the volatility and biological properties of plumbagin make this a suitable compound for defense mechanisms in some plants [15]. It is important to mention that plumbagin was the quinone we easily identified in the hexanic extracts after treatment with KOH/HCl because it crystalised as red needles from fraction C. This method has been proved in the separation of

quinones from oil mixtures present in the stem bark extracts of *Diospyros cuneata* and in the separation of the anthraquinones aloesaponarin II and laccaic acid D methyl ester from hexanic extracts of roots of *Aloe vera* (personal experience).

In conclusion, the four methods employed in combination with KOH/HCl allowed the separation of slightly polar quinones from terpenoids and fatty ester mixtures from the stem bark extract of *D.anisandra*. The combined methods allow for the separation and further purification and identification of quinones by GC-MS and NMR analysis. Regarding the isolation of slightly polar volatile quinones, static maceration is one of the better methods. Further experiments on the antimycobacterial activities of each quinone are in progress.

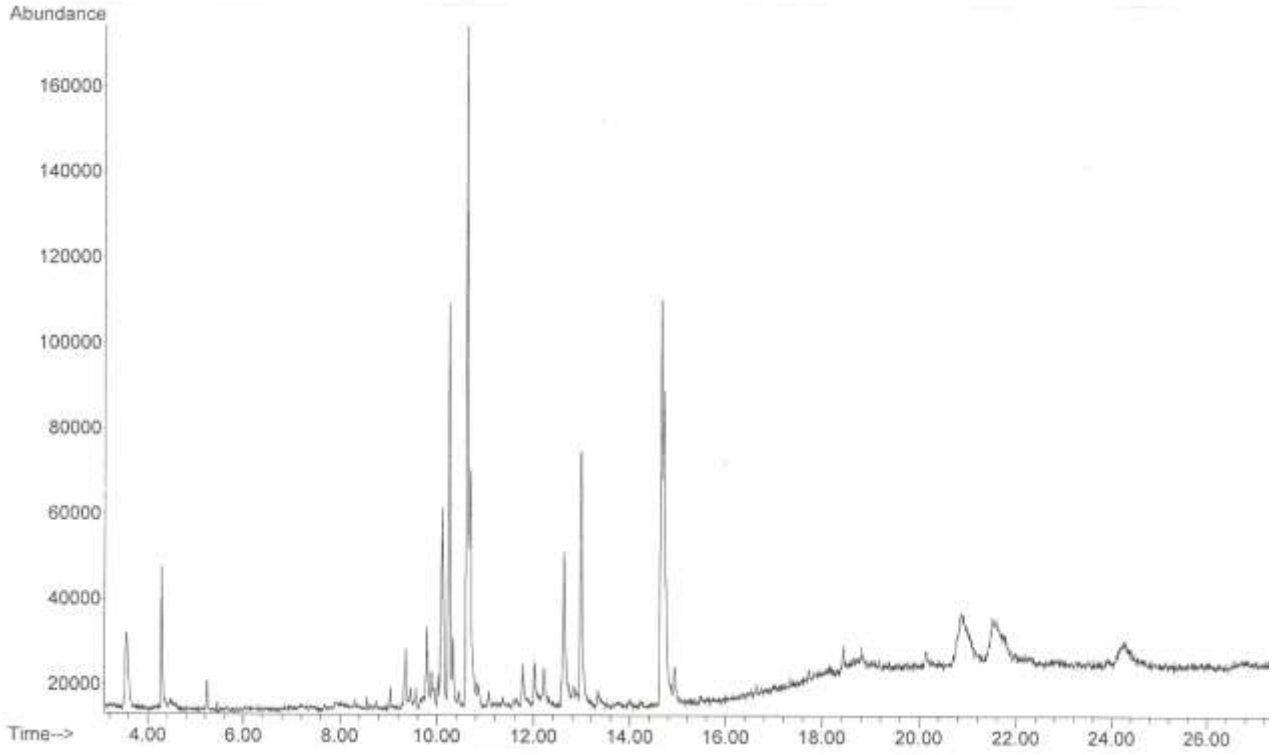

Fig. 3: Chromatogram of the terpene/fatty esther fraction of *Diospyros anisandra* obtained by static maceration after KOH/HCl treatment.

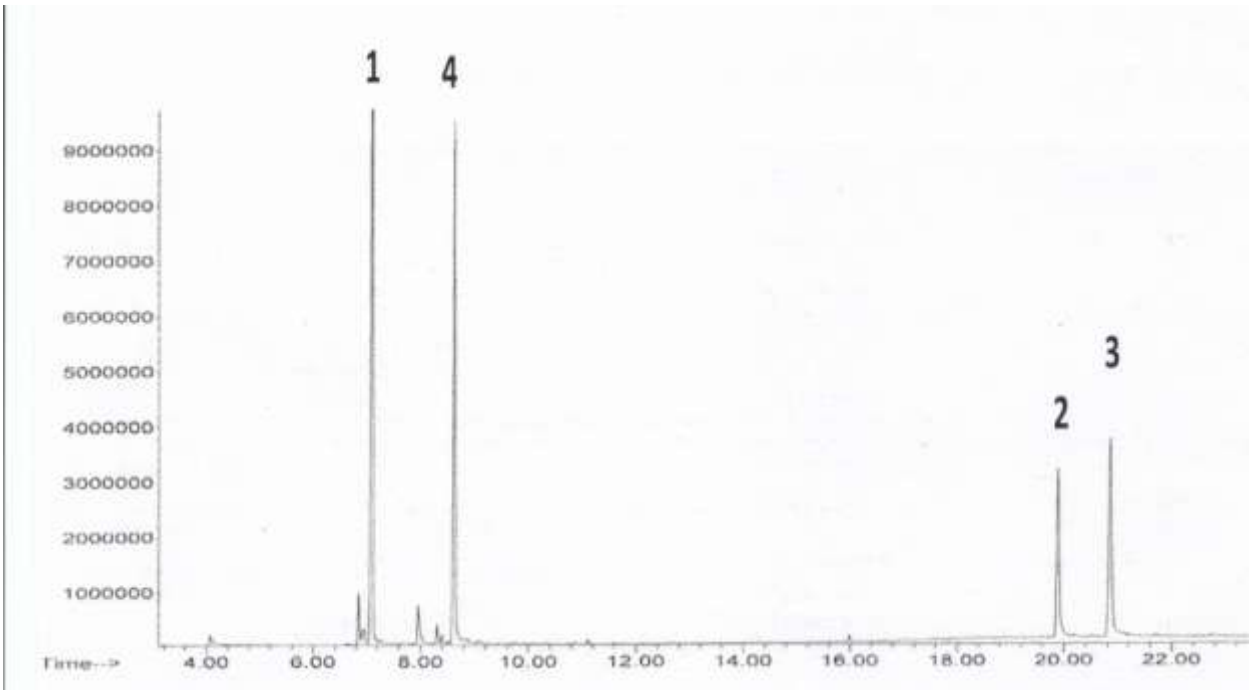

Fig. 4: Chromatogram of the quinone fraction of *Diospyros anisandra* obtained by static maceration after KOH/HCl treatment. The peaks are identified as follows: plumbagin (1), maritinone (2), 3,3'-biplumbagin (3) and droserone (4).

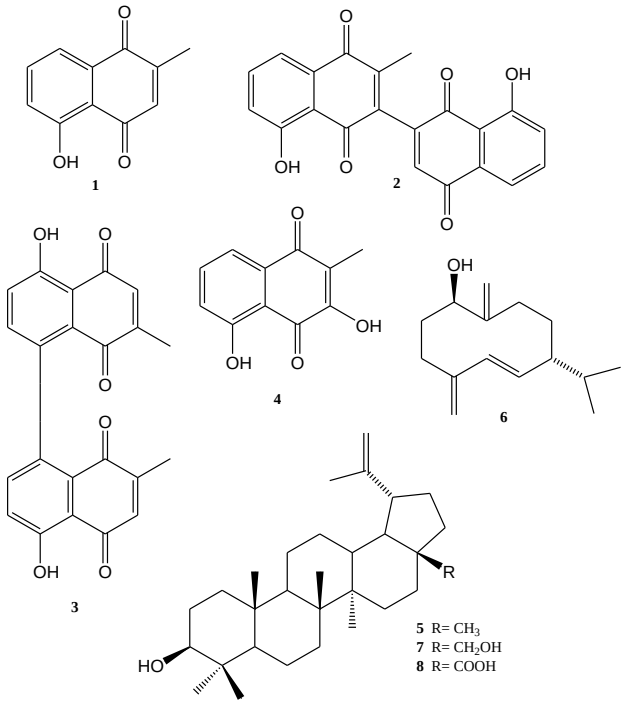

Fig. 5: Quinones and terpenes isolated from the stem bark extract of *Diospyros anisandra*.

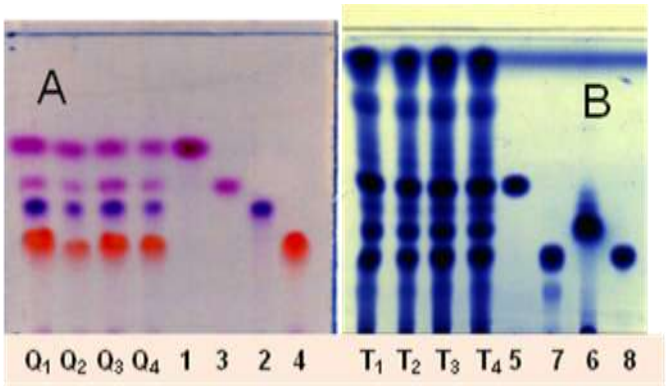

Fig. 6: Thin-layer chromatography of the quinone-rich fraction (A) and terpene-rich fraction (B) after treating the hexanic extracts with KOH/HCl obtained by static maceration (Q_1,T_1), dynamic maceration (Q_2,T_2), soxhlet extraction (Q_3,T_3), and ultrasound-assisted extraction (Q_4,T_4) reveled with 5% KOH in ethanol (A) and revealed with phosphomolybdic acid (B) together with the pure compounds plumbagin (1), maritinone (2), 3'3-biplumbagin (3), droserone (4), lupeol (5), 4(15),5E, 10 (14)-germacratrien-1β-ol (6), betulin (7) and betulinic acid (8). TLC system *n*-hexane:acetone 8:2.

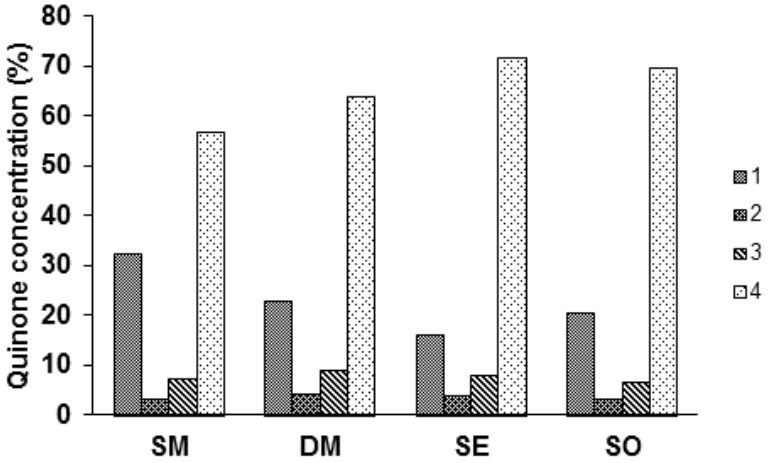

Fig. 7: Concentration of each quinone (%) isolated from the *n*-hexanic extracts obtained by static maceration (SM), dynamic maceration (DM), Soxhlet extraction (SE), and ultrasound-assisted extraction (SO). Pure compounds are identified as 1=plumbagin, 2=maritinone, 3=3'3-biplumbagin and 4=droserone.

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