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Chromatographic isolation and estimation of zanthoxylol: an antisickling agent from the roots of *Zanthoxylum* species

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Abstract: The roots of four species of *Zanthoxylum* were examined for the presence of zanthoxylol (2-dimethylallyl-4-(3-hydroxy-propyl)phenol) but only *Z. zanthoxyloides* was found to contain this substance. Purification was effected using Sephadex ion-exchange resins; isolation and quantification were performed on reversed-phase HPLC columns. The identity was confirmed by UV, IR, NMR, GC and MS analysis. The zanthoxylol content was calculated as *p*-hydroxybenzoic acid. The possible chemotaxonomic value of the zanthoxylol content in *Zanthoxylum* species is speculated.

Keywords: Zanthoxylum; *zanthoxylol; antisickling agent; reversed-phase high-performance liquid chromatography.*

Introduction

The genus *Zanthoxylum* Linn. (*Fagara* Linn.) (Rutaceae) is widely distributed in Nigeria where the roots are used as chewing sticks. Some members of this genus have been implicated in the reversal of sickled red blood cells to normal and the inhibition of sickling of homozygous HbSS because of their content of acidic phenols [1–3] as well as zanthoxylol, a non-acidic phenol [4]. Eshiett and Taylor [5] have reported the presence of zanthoxylol in the heartwood of *Fagara zanthoxyloides (Z. zanthoxyloides)*. There are many species of *Zanthoxylum* in Nigeria; for antisickling formulations, it is necessary to know which species could yield zanthoxylol and how much. The present work confirms the presence of and quantifies this compound in the root of *Z. zanthoxyloides*.

Experimental

Plant material

The roots of *Z. zanthoxyloides* (Lam.) Waterm., *Z. lemairel* (De Wild.) Waterm., *Z. leprieurii* (Guill. et Perr.) Waterm. and *Z. tessmanii* (Engl.) Waterm. were collected from their different locations in Nigeria during the rainy season (May–August) and

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compared with the herbarium specimens previously deposited in the Botany Department Herbarium, University of Ife. Their identities were also confirmed in the Forestry Research Institute of Nigeria, Ibadan. The roots were chopped, dried in an oven at 60°C and powdered before extraction.

Other materials

Sephadex ion-exchange resins (Pharmacia) were used; since an authentic zanthoxylol was not available, *p*-hydroxybenzoic acid (Fluka) was used as reference standard for the calibration graph.

Extraction of plant sample and ion-exchange chromatography

0.1 kg of the dry powdered root of each of the four *Zanthoxylum* species was extracted for 48 h in a Soxhlet apparatus with ethyl acetate. The solvent was removed completely by evaporation *in vacuo* and the residue was redissolved in 80% ethanol; this solution was evaporated *in vacuo* leaving approximately 10 ml of aqueous extract. The aqueous extract was then passed through a 200 × 15 mm i.d. column packed with SP-C25 Sephadex ion-exchange-resin (hydrogen form). Basic materials in the extracts were adsorbed but phenolics were eluted with water until the last column effluent did not form a colour with a solution of ferric chloride in pyridine [5]. The water eluate from this column, after concentration *in vacuo*, was transferred to a 200 × 15 mm i.d. column packed with Sephadex DEAE-A25 (acetate form).

Phenolic carboxylic acids were strongly adsorbed on the material in this latter column while non-carboxylic phenols were freely eluted with 0.1 M NH_4OAc until the last column effluent did not react with a solution of ferric chloride in pyridine. The NH_4OAc was removed *in vacuo* from the eluate and the residue was redissolved in 100 ml of ethanol to give the "purified extract".

Analytical HPLC

The analysis was performed on a Hewlett–Packard 1080B liquid chromatograph equipped with a variable wavelength detector and an automatic injector. A 5 μl sample of each of six different standard dilutions from a 1% (m/v) solution of the reference sample of *p*-hydroxybenzoic acid was injected in duplicate on a 250 × 4.6 mm i.d. metal column packed with Lichrosorb 10RP-8 (Merck); the peak areas were obtained from the recorder–integrator coupled to the UV detector at 280 nm. Solvent A was water acidified to pH 3 with acetic acid; solvent B was methanol. Gradient elution was at 4 ml/min from 5% B to 80% B in 40 min. The mean values of the peak areas were plotted against the amounts (mg) of *p*-hydroxybenzoic acid for use as a calibration graph. Samples (10 μl) of the "purified extract" obtained from each of the *Zanthoxylum* species were similarly injected but in quadruplicate; the calibration graph was used to derive the amount of zanthoxylol (as *p*-hydroxybenzoic acid) from the mean peak areas.

Preparative HPLC

Replicate injections of the "purified extract" were made on a 250 × 22.4 mm i.d. stainless-steel column packed with Lichrosorb 10RP-8 (Merck); isocratic elution with acetic acid (pH 3)–methanol (20:80, v/v) was carried out. The peak corresponding to R23 was collected and the fraction was evaporated to dryness. The dry residue (about 48 mg) was further examined using various chromatographic and spectroscopic techniques. A known quantity of the residue was dissolved in ethanol to give a standard solution of

compound R23 and 5 μl of this solution was injected in duplicate on a HPLC column (as described for Analytical HPLC) and the amount of zanthoxylol (as *p*-hydroxybenzoic acid) derived from the mean peak area by reference to the calibration graph. The HPLC of the eluted compound R23 gave a single peak without any interference; the base line was smoother than that for the "purified extract" but the overall results for content of zanthoxylol were not significantly different.

Qualitative gas chromatography

A 100 μl portion of the solution of *p*-hydroxybenzoic acid or the solution of the "purified extract" or an ethanolic solution of the compound R23, isolated by preparative HPLC (as above), was evaporated separately and dried; each residue was dissolved in 100 μl of dry pyridine with dodecane as the internal standard. A 100 μl sample of BSTFA containing 1% TMCS was added and the mixture was heated at 90°C for 20 min; then 2 μl of this mixture was injected in duplicate on a Varian 3700 chromatograph with a FID detector and fitted with a 500 × 2 mm i.d. metal column packed with Chromosorb G HP 100/120 (5% OV-101). Temperature gradient elution was from 120°C to 280°C at 60°C/min. Nitrogen flow-rate was 20 ml/min. Retention times were recorded in min. The GC equipment was coupled to a Ribermag mass spectrometer (see below) for GC–MS qualitative analysis.

Mass spectroscopy

The mass spectral data of compound R23 (isolated by preparative HPLC) as well as that of the corresponding peak, selected from the gas chromatogram, were separately obtained on a Riber R10-10B quadrupole mass spectrometer operating on-line with the gas chromatograph. It was equipped with a System Industries 150 interface, a PDP8/a computer and a CI/D, EI source. Ionization was accomplished at 70 eV; the ion source was at a constant pressure of 2.6×10^{-7} bar.

N.M.R.

Compound R23 was dissolved in CD Cl_3 and then subjected to 100 MH_z proton N.M.R. (Jeol apparatus) analysis using tetramethylsilane as the internal standard. The N.M.R. spectrum was compared with published data for zanthoxylol [5].

Results and Discussion

HPLC analysis of the reference substance *p*-hydroxybenzoic acid gave a single peak at 5.61 min whereas HPLC of the "purified extract" of each of the *Zanthoxylum* species roots showed that all the major UV absorbing compounds were present in that fraction. For example, that of *Z. zanthoxyloides* gave four major peaks, R3, R14, R16 and R23 (retention times 3.11, 14.13, 16.03 and 23.52 min, respectively) (Fig. 1). When these peaks were individually collected from the preparative HPLC column, only compound R23 was pure when re-chromatographed on HPLC; the GC of the underivatized compound R23 gave a single peak (retention time = 15 min); GC of its TMS derivative gave a single peak (retention time = 40 min); and TLC (silica gel G) gave a single spot. GC of the TMS derivative of *p*-hydroxybenzoic acid gave a single peak (retention time = 11 min).

The GC peak (broad with a shoulder and tailing) obtained from the underivatized R23 sample was inadequate for reliable quantitative analysis and, although the TMS

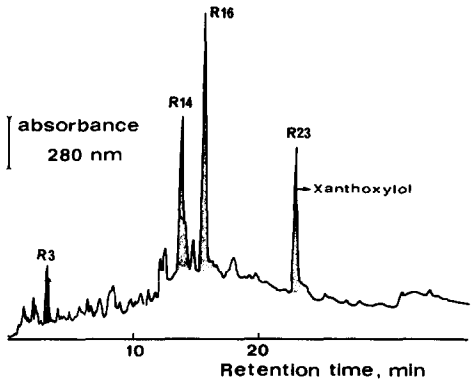


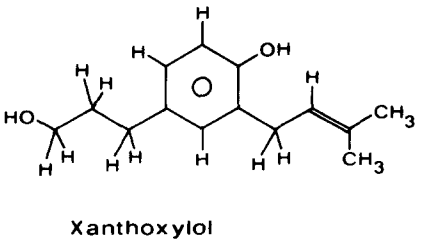
Figure 1
HPLC of the UV-absorbing components of the "purified extract" of *Z. zanthoxyloides* root. For details of HPLC see text.

derivative gave an excellent peak, the retention time (40 min) was too long. Therefore, the HPLC procedure was chosen for quantitative analysis; GC could serve as a supportive qualitative method for zanthoxylol.

The on-line recording of the UV spectrum of compound R23 gave a single band at 280 nm. Its ^{1}H NMR spectrum showed the following resonances: a singlet at 1.77 ppm (two $-CH_3$ groups); a multiplet around 1.90 ppm ($-CH_2-CH_2-CH_2-$ Ph); a triplet at 2.54, 2.62, 2.69 ppm ($-CH_2-CH_2-$ Ph); a doublet at 3.29, 3.36 ppm (– Ph – CH_2 – CH =); a triplet at 3.61, 3.67, 3.73 ppm ($-CH_2-CH_2-$ OH); a triplet at 5.20, 5.28, 5.36 ppm ($-CH_2-CH=C$); resonances from three aromatic protons at 6.7 ppm (doublet), at 6.9 ppm (doublet) and at 7.3 ppm (singlet). Its IR spectrum (Nujol) confirmed the presence of a hydroxyl group near 3400 cm^{-1} and an aromatic ring near 1600 cm^{-1} but there was no signal near 1700 cm^{-1} (carbonyl group absent). GC–MS analysis of the underivatized compound R23 gave peaks at the following *m/z* and relative intensity values: 220 (M^+, 94%); 205 ($M^+ - 15$, 4.6%); 175 (base peak, 100%); 165 (61%); 121 (81%). It is proposed that the most abundant ion (*m/z* 175) could be attributed to the removal of part of the 3-hydroxypropyl moiety, i.e. ($\dot{C}H_2CH_2OH$) from the parent ion. GC/MS analysis of the trimethylsilyl-derivatized R23 gave peaks at *m/z* values 364 ($M^+ - 15$, 5%); 274 (33%); 259 (45%); 247 (30%); 205 (92%). The high resolution mass spectrum of the underivatized R23 gave $C_{14}H_{20}O_2$ with an exact molecular mass of 220.1390.

The NMR results and the elemental analysis of Eshiett and Taylor [5] for zanthoxylol, were in agreement with the findings in the present work; furthermore, on the basis of the UV, IR, NMR, GC/MS and high resolution mass spectral data, compound R23 was identified as zanthoxylol (Fig. 2).

The HPLC procedure is adequate for the isolation and quantification of zanthoxylol from *Zanthoxylum* species. Both the gradient and isocratic elution techniques gave



Xanthoxylol

Figure 2
The structure of compound R23 identified as zanthoxylol, from *Z. zanthoxyloides* root extract.

similar results but because of its simplicity, the technique is recommended for use in the routine detection and comparative estimation of zanthoxylol in plant extracts. The SP-25 exchange-resin absorbs most of the alkaloids present in *Zanthoxylum* root crude extract [6] whereas the non-alkaloidal phenolics [1, 2] are freely eluted. The DEAE-A25 exchange-resin similarly absorbs the carboxylic acid phenols leaving only the non-carboxylic acid phenols (including zanthoxylol) to be eluted with 0.1 M NH_4OAc.

For the *Z. zanthoxyloides* root, when ten separate 5-μl injections of the "purified extract" were made on HPLC, the mean amount of zanthoxylol (as *p*-hydroxybenzoic acid) per 100 g of dry root was 57.50 mg with a standard deviation of ±0.13 mg; amounts of the unidentified compounds were R3 (22.02 mg), R14 (77,11 mg) and R16 (102.23 mg). These quantitative measurements have been based on *p*-hydroxybenzoic acid as the reference standard and thus only represent approximate rather than absolute values. Despite these limitations, the method can still serve in the quality control, detection and relative estimation of zanthoxylol in commercial crude drug samples of *Zanthoxylum*; thus the method can be used to compare the amounts of the compound in the various plant species. Zanthoxylol was absent in the three other species investigated in the present work; this result supports the findings of Eshiett and Taylor [5, 7] who have reported the absence of zanthoxylol in the woods of both *Z. lemairei* and *Z. leprieurri*. To the authors' knowledge, this report is the first to be published on the use of HPLC in the isolation and quantification of zanthoxylol in the genus *Zanthoxylum*.

Adesanya and Sofowora [3] have reported recently that *Z. zanthoxyloides* possessed the highest antisickling activity of all species examined in their work. Although the authors of the present work have reported earlier that all these species contained acids in various amounts [8], the presence of zanthoxylol in *Z. zanthoxyloides* and its absence in the other three species may explain the higher antisickling activity of *Z. zanthoxyloides*. Zanthoxylol will therefore confer an additional antisickling effect on any species in which it is present. The possibility of using this compound also as a chemotaxonomic index for some taxonomically confused members of the genus, *Zanthoxylum*, is being investigated.

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