International Journal of Pharmaceutics, 53 (1989) R1–R3
Elsevier

IJP 01906

Rapid Communication

Chromatographic analysis of antisickling compounds in *Zanthoxylum* species

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(Received 12 May 1989)
(Modified version received 26 May 1989)
(Accepted 1 June 1989)

Key words: Zanthoxylum species; Antisickling compound; Chromatographic analysis

Summary

Five species of *Zanthoxylum* were found to contain antisickling phenolic acids in varying quantities, using ion-exchange columns, spectroscopy and reversed-phase HPLC. Application of the findings to the quality control of raw materials intended for antisickling preparations is proposed.

Z. zanthoxyloides (Lam.) Waterm. formerly *Fagara zanthoxyloides* Lam. (Rutaceae) is the best known of about 10 species of *Zanthoxylum* growing in Nigeria where they are used as chewing sticks. Its root's aqueous extract has been reported to possess antisickling properties on sickle cell haemoglobin SS (Sofowora and Isaacs-Sodeye, 1971) attributable to the presence of phenolic acids (Sofowora et al., 1975). Thus, vanillic and *p*-hydroxybenzoic acids were found to inhibit and reverse sickled cells in vitro, respectively (Adesanya and Sofowora, 1983). The total (phenolic and aliphatic) acid contents in 6 of the Nigerian *Zanthoxylum* species have been estimated by a non-specific colorimetric method (Elujoba and Sofowora, 1977). An assay method, quantifying only the antisickling acids individually and also useful for predicting bioactivity as well as for quality control assessments, was soon found to be necessary. The present report provides a procedure for detecting the phenolic acids in the root extracts of 4 *Zanthoxylum* species by GCMS and quantifying them individually by HPLC. This will permit an estimation to be made of the total antisickling effect (reversal or inhibitory) expected from any plant extract containing phenolic acids in appreciable quantities. Also one of the phenolic acids (vanillic) was characterized from the leaves of two species of *Zanthoxylum*.

The root. The chopped, dried roots of *Zanthoxylum lemairei* (DeWild) Waterm., *Z. leprieurii* (Guill. et Perr.) Waterm., *Z. tessmanii* (Eng.) Waterm., and *Z. zanthoxyloides* (Lam.) Waterm., collected in Nigeria and authenticated as before (Elujoba and Sofowora, 1977), were each extracted exhaustively with diethyl ether which was concentrated and partitioned with 0.05 M

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NH_4OH. The aqueous fraction was processed on a Sephadex DEAE column (10 × 1.5 cm i.d.) by eluting with 0.01 M NH_4OAc (F1) followed by 0.1 N HCl (F2). Fraction F2 was extracted into ethyl acetate, evaporated to dryness and redissolved in 1 ml ethanol to give F3. 100 μl of this solution was injected semi-preparatively on a high-performance liquid chromatograph (Hewlett Parkard 1080B) with Lichrosorb RP8 column (25 cm × 4.6 mm i.d.). Solvent system was 0.01 M CH_3COOH–MeOH, gradient elution at 4 ml/min from 5% to 80% MeOH in 40 min, UV detection at 280 nm. Fractions were collected and reinjected on a Lichrosorb 10 DIOL column (25 cm × 4.6 mm i.d.) with hexane–acetic acid (99.5 : 0.5 v/v) and propanol, gradient elution from 5% to 20% propanol in 20 min. The phenolic acids were estimated from the calibration graphs of the appropriate authentic samples. Another 100 μl of fraction F3 was evaporated to dryness in vacuo and silylated with pyridine and BSTFA containing 1% TMCS for 20 min at 90 °C and analysed on a Varian 3700 gas chromatograph, FID detector, 0.5 m × 2 mm i.d. metal column with Chromosorb HP 100/120 (OV-101). Temp. gradient from 120 to 280 °C at 6 °C/min; nitrogen at 20 ml/min. A similar condition was used for the subsequent GCMS analysis.

The leaf. The dried, powdered leaves of *Zanthoxylum rubescence* (Planch ex Hook) Waterm. and *Z. zanthoxyloides* were each extracted with distilled water (1 litre), concentrated in vacuo (250 ml) and shaken with diethyl ether (200 ml × 4). The ether fractions were bulked and evaporated to dryness (antisickling fraction; Sofowora et al., 1975). This was redissolved in 50% ethanol and on evaporation left an aqueous suspension which was first passed through a Sephadex SP-C25 column (7 × 3 cm i.d.) and eluted with water. The aqueous eluate was treated as above to obtain F1, F2 and F3. The F3 fraction was subjected to PTLC on silica gel with $CHCl_3$–AcOH (9 : 1); $R_f = 0.72$ band was recovered into methanol which was evaporated. Addition of acetone to the residue gave a whitish precipitate which was filtered off while the filtrate gave a whitish solid (compound **V**) on evaporation to dryness. Compound **V** was similarly treated on HPLC, GLC, GCMS and also analysed on UV and IR.

TABLE 1

Concentration (ppm) and chromatographic retention times of phenolic acids in Zanthoxylum species roots

Zanthoxylum species	Protocatechuic acid	*p*-OH-benzoic acid	Vanillic acid	Syringic acid
Z. lemairei	4.7	24.4	17.5	18.0
Z. leprieurii	0.4	8.1	3.0	9.7
Z. tessmanii	0.0	7.0	19.6	2.6
Z. zanthoxyloides	0.0	10.0	13.3	1.8
RP column (t_R, min)		7.7	9.5	19.8
DIOL column (t_R, min)		4.5	5.0	6.9

A wide variety of chromatographic systems for the analysis of phenolic substances had been reported for TLC (Van Sumere et al., 1965; Jangaard, 1970), PC (Reio, 1958) and reversed-phase HPLC (Court, 1977; Murphy and Stutte, 1978; Wulf and Nagels, 1976). With regard to the *Zanthoxylum* phenolics, we have found TLC and PC useful only in the qualitative application but the quantitative analysis was more accurately performed by HPLC than TLC, PC or our previous colorimetric method (Elujoba and Sofowora, 1977). For the efficient separation and quantification of the individual phenolic acids without undue interference, the coupling of two HPLC columns in series was found to be necessary in the exercise. The GCMS analysis of the purified extracts of *Zanthoxylum* roots in the present work, revealed the presence of *p*-OH-benzoic, ferulic, vanillic, syringic, protocatechuic and *p*-coumaric acids. The HPLC assay shows that these acids were present in varying concentrations in all the species studied (Table 1). Ferulic and *p*-coumaric acids were detected in trace amounts in the roots tested. The presence of syringic, ferulic, *p*-coumaric and protocatechuic acids is being reported for the first time in these or any species of *Zanthoxylum* and the present assay procedure is a major advancement on our earlier colorimetric method (Elujoba and Sofowora, 1977) for the total acids in the roots. However, both methods agreed that *Z. lemairei* root contained

the highest concentration of these acids. The latest method has thus provided a means of calculating the total amounts of sickling reversal or sickling inhibitory activity present in any given *Zanthoxylum* root extract. This is possible by a summation of the units of antisickling activities (Adesanya and Sofowora, 1983) of each phenolic acid so quantified using the procedure now described.

Compound **V**, isolated from the leaves of *Z. rubescens* and *Z. zanthoxyloides*, was characterized by TLC ($R_f = 0.72$); UV(MeOH) = 289, 256 nm; IR(nujol) = 3540, 1730, 1600, 1580 cm^{-1}; GLC(t_R = 23 min); HPLC(RP column, $t_R = 9.18$ min); GCMS trimethylsilyl derivatives: m/z 312(M +), 297(M-15), 253(M-59), 223(M-89), 73(100%); the computer search for m/z 312 suggested vanillic acid trimethylsilyl whose authentic sample gave similar spectral and chromatographic chracteristics to compound **V**. If the antisickling index of the leaf is found to be comparable to that of the root of these plants, it will be more economical to earmark the leaf as the prefered raw material for antisickling preparations because of the danger of eradication by continuous root collection.

Both the qualitative and quantitative procedures in this work can serve as efficient quality control facilities for monitoring both the raw materials and finished products for sickle cell anaemia therapy.

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