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Short communication

Identification and quantitation of the ingredients in a counterfeit Vietnamese herbal medicine against rheumatic diseases

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ABSTRACT

Counterfeit and/or illegally manufactured drugs and herbal medicines are becoming an increasing problem throughout the world. Internet sales simplify distribution and payment of these falsified drugs. Here we report on a Vietnamese herbal medicine, which was advertised for treatment of rheumatic disease from a religious Vietnamese healer. By means of NMR and LC/MS we found 863 mg acetaminophen, 262 mg sulfamethoxazole, 42 mg indomethacin and less than 1% trimethoprim in a sachet of 2.617 g powder content, in addition to some cinnamon bark and phosphate.

1. Introduction

People suffering from rheumatic diseases are tempted to buy herbal medicines because the antirheumatic therapy with chemical drugs is accompanied by frequently occurring strong side effects, e.g. due to gastro-intestinal bleeding. Visiting the internet reveals a huge selection of herbals against rheumatoid arthritis form Asian countries, such as China, India (TCM, Ayurvedic, Unani or Tibbi), and Korea [1–3]. For many Indian herbals, the pharmacological activity is well-described, e.g. [4]. Other herbals are of low quality and illegal, and possibly dangerous for the patients [5]. In addition, a lot of so-called herbals, available in the internet, contain chemically defined drugs beside herbals [6], e.g. in the field of lifestyle drugs such as tadalafil [7] and sildenafil [8,9]. Here, the chemical drug is mostly not labeled.

Counterfeit and/or illegally manufactured drugs and herbal medicines are becoming an increasing problem throughout the world. The problem is well documented in the literature [10]. Unravelling the counterfeiting by identification of the ingredients of the (herbal) drugs is always a challenge. Mostly a combination of many spectroscopic techniques such as high performance liquid chromatography hyphenated with mass spectrometry and NMR spectroscopy is necessary for identification and quantification of the active pharmaceutical components [11,12] in addition to special techniques as DOSY ^{1}H NMR [13].

Recently, we got such an herbal drug from a female rheumatic patient who bought the medicine from a religious Vietnamese healer. It was advertised as 100% natural and accompanied by the advice to take one sachet per day. A sachet contained 2.6 g of a brownish powder, smelling of cinnamon. Unfortunately, no labeling was found. For identification and quantification of the ingredients HPLC–UV and –MS, NMR and IR experiments as well as some inorganic tests were applied, in addition light microscope studies to elucidate the herbal ingredients.

2. Material and methods

2.1. Chemicals

HPLC grade acetonitrile and methanol were purchased from VWR (Darmstadt, Germany), acetic acid ACS reagent ≥99.7% from Sigma-Aldrich (Schnelldorf, Germany), isopropyl alcohol ≥99.7% from Bernd Kraft (Duisburg, Germany) and hexadeuteriodimethyl sulfoxide (DMSO-d_6, 99.8% D) from Euriso-top (Saarbrücken, Germany). HPLC grade water was obtained by in-house Millipore system from Merck (Darmstadt, Germany). As reference standards, acetaminophen was purchased from Rhodia Organique (Venisseux, France), trimethoprim from Procter&Gamble (Cincinnati, USA), sulfamethoxazole from Berlin-Chemie (Berlin, Germany) and indomethacin from Sigma-Aldrich (Schnelldorf, Germany).

Abbreviations: ^{1}H NMR spectroscopy, proton nuclear magnetic resonance; NSAID, non-steroidal anti-inflammatory drug.

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2.2. NMR spectroscopy

NMR measurements were performed on a Bruker Avance III 400 MHz spectrometer (Bruker BioSpin, Karlsruhe, Germany) with a PABBI 1H/D-BB 5 mm probe head, and data processing with the TopSpin 3.0 software. Depending on the concentration the number of scans varied from 128 to 13k for ^{1}H NMR and 512 to 12k for ^{13}C NMR measurements. The standard measurements ^{1}H NMR were performed at 300 K, flip angle 30°, spectral width of 20.55 ppm, transmitter offset of 6.175 ppm, acquisition time 3.985 s followed by a relaxation delay varied from 1 to 20 s. 64k data points were collected at a spinning frequency of 20 Hz. Processing parameters were set to an exponential line broadening window function of 0.3 Hz, an automatic baseline correction and manual phasing. The spectra were referenced to the residual solvent signal of DMSO (2.5 ppm/39.52 ppm). For structure elucidation a full characterization was performed consisting of standard ^{1}H, ^{13}C, distortionless enhancement by polarization transfer (DEPT-135), correlated spectroscop**y** (H-H-COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments. Standard 5 mm NMR tubes (ST 500) were purchased from Norell (Landisville, USA).

For a first NMR screen of the entire sample, 20 mg were dissolved in 700 μl DMSO-d_6. For structure elucidation the respective fractions of 10 preparative HPLC runs (see below) were evaporated in vacuo and the residue was dissolved in 550 μl DMSO-d_6.

2.3. HPLC chromatography

Analytical and preparative HPLC analyses were performed using an Agilent Technologies 1100 series systems (Waldbronn, Germany). The analytical system consisted of a binary pump, a degasser, an autosampler, a column thermostat and a diode-array UV detector. The preparative system consisted of a binary pump, an autosampler, fraction collector and a diode-array UV detector without an degasser, therefore the solvents were degassed by Sonorex bath Bandelin (Berlin, Germany) for 30 min. The LC–MS system consisted of a 1100 series HPLC, ESI source and a LC/MSD Trap of Agilent Technologies.

A Nucleodur Sphinx RP (150 × 4.6 mm, 5 μm) column was used for analytical purposes and the column a Nucleodur Sphinx RP (125 × 10 mm, 5 μm) for preparative isolation of the components. HPLC vials and caps were purchased from Phenomenex (Aschaffenburg, Germany). The samples were centrifuged with 13 000 rpm 10 min by an EBA 12 centrifuge of Hettich (Tuttlingen, Germany).

The separation was carried out by means of the method reported by Panusa et al. [12] using a Nucleodur Sphinx RP column and a gradient of water (A) and acetonitrile (B) each with acetic acid 0.1% and applying a wavelength of 254 nm. The elution started isocratic 85:15 (A:B, v/v) for 3 min, to 70:30 (A:B, v/v) in 7 min and from 70:30 to 10:90 (A:B, v/v) in 20 min. After each run the column was washed with 100% B for 5 min and then conditioned with 85:15 (A:B, v/v) for 10 min. The injection volume was 5 μl and the column thermostat was kept at 30 °C. This method was scaled up with a flow of 4.6 ml min^{-1} for preparative HPLC.

For structure elucidation purposes the HPLC – using the Panusa method – was hyphenated to an ESI–MS detector: the parameter for positive mode and negative ionization mode was set to 350 °C drying temperature, 70.00 psi nebulizer, 12.0 l min^{-1} drying gas (nitrogen) and a scanning range from 50–800 *m/z*.

2.4. IR spectroscopy

For IR studies the test compound was washed with isopropyl alcohol to separate the inorganic material and organic molecules. The spectra were conducted on Jasco FT/IR-6100 spectrometer from Jasco (Gross-Umstadt, Germany).

2.5. Spectroscopic data of the ingredients

2.5.1. Acetaminophen

^{1}H-NMR (DMSO-d_6, δ (ppm) J (Hz)): 9.62 (s, 1*H*, N**H**), 9.11 (s, 1*H*, O**H**), 7.33 (m, 2*H*, **H**-arom), 6.66 (m, 2*H*, **H**-arom), 1.97 (s, 3*H*, C**H$_3$**).

2.5.2. Trimethoprim

^{1}H-NMR (DMSO-d_6, δ (ppm), J (Hz)): 7.52 (s, 1*H*, **H**-arom), 6.55 (s, 2*H*, **H**-arom), 6.09 (s, 2*H*, N**H$_2$**), 5.71 (s, 2*H*, N**H$_2$**), 3.72 (s, 6*H*, 2×OC**H$_3$**), 3.62 (s, 3*H*, OC**H$_3$**), 3.53 (s, 2*H*, C**H$_2$**).

2.5.3. Sulfamethoxazole

^{1}H-NMR (DMSO-d_6, δ (ppm), J (Hz)): 10.9 (s, 1*H*, N**H**), 7.45 (m, 2*H*, **H**-arom), 6.56 (m, 2*H*, **H**-arom), 6.09 (d, 1*H*, ^{4}J = 0.8 Hz, **H**-oxazole), 6.06 (s, 2*H*, N**H$_2$**), 2.28 (d, 3*H*, ^{4}J = 0.8 Hz, C**H$_3$**).

2.5.4. Indomethacin

^{1}H-NMR (DMSO-d_6, δ (ppm), J (Hz): 12.35 (s, 1*H*, COO**H**), 7.66 (m, 4*H*, **H**-arom), 7.04 (d, 1*H*, ^{4}J = 2.5 Hz **H**-arom), 6.93 (d, 1*H*, ^{3}J = 9.0 Hz, **H**-arom), 6.70 (dd, 1*H*, ^{3}J = 9.0 Hz, ^{4}J = 2.5 Hz, **H**-arom), 3.75 (s, 3*H*,-OC**H$_3$**), 3.66 (s, 2*H*, C**H$_2$**COOH), 2.20 (s, 3*H*, C**H$_3$**).

2.6. Quantification of the active ingredients—Standard addition

One sachet herbal mix contained 2.617 g brownish powder. The powder was grounded in a mortar for HPLC and NMR sample preparation.

The concentration of herbal mix was 1 mg ml^{-1} for preliminary HPLC and MS test, 100 mg ml^{-1} for preparative HPLC and 0.8522 mg ml^{-1} for analytical HPLC dissolved in methanol by 10 min sonication and then centrifuged at 13 000 rpm for 10 min. For standard addition the concentration was 214.79 mg acetaminophen, 68.23 mg sulfamethoxazole and 19.04 mg indomethacin dissolved in 250 ml methanol. To the test solution of 0.8522 mg ml^{-1} test compound, 100, 200, 300, 400, 500 μl standard solutions were added.

2.7. Test for phosphate

One hundred milligram test compound was washed with isopropyl alcohol to separate the inorganic material and organic molecules. The residue was dried for 24 h over silica gel in vacuo and 20 mg inorganic material was mixed with 1 ml nitric acid 60% and 20 mg ammonium molybdate. The solution was heated up for 1 min and after 20 min cooling to room temperature a yellow salt precipitated.

2.8. Microscopic inspection

The herbal mixture was examined by a CX41 microscope from Olympus (Hamburg, Germany), and the microscope images were recorded by an EC3 microscope camera from Leica (Nidau, Switzerland).

3. Results and discussion

3.1. Identification

In order to get a general idea about the ingredients of the sachet the powder was pestle to get a uniform powder. 20 mg of this powder was dissolved in DMSO-d_6 and a ^{1}H NMR spectrum was measured (see Fig. 2). The NMR spectrum was dominated by the signals of acetaminophen (see Fig. 1), i.e. two multiplets ca. at δ = 6.66