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Identification of individual herbal drugs in tea mixtures using restriction analysis of ITS DNA and real-time PCR

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We have studied a sedative tea made of Valerianae radix (*Valeriana officinalis* L.), Lupuli strobuli (*Humulus lupulus* L.), Melissae folium (*Melissa officinalis* L.) and Menthae piperitae folium (*Mentha piperita* L.). In order to identify the constituent drugs a method was established involving amplification of the internal transcribed spacers (ITS) region of nuclear ribosomal DNA on the basis of restriction analysis and real-time PCR. ITS regions of individual drugs were amplified and sequenced. Restriction analysis was performed with selected restriction endonucleases Nae I, PshA I and Xcm I. Real-time PCR was carried out, using primers specifically designed for each individual herbal drug. Real-time PCR proved to be a method for identifying individual herbal drugs in a tea mixture with a single DNA extraction in a single PCR run, since its limit of detection is lower than that for restriction analysis.

1. Introduction

In recent years, interest in herbal medicines has been increasing in the developed world. The relative popularity of therapies differs between countries, but it is estimated that complementary therapies are used by 20% to almost 50% of the population (Fisher and Ward 1994).

Herbal teas, one of the oldest galenic preparations, are by definition preparations made of one herbal drug or drug mixtures (Bisset and Wichtl 2001). The mixtures are either prepared *"in situ"* in the pharmacy or industrially, comprising several drugs often belonging to the same indication group. Moreover, in some teas, supplementary drugs are added to the principal drugs that supplement the action or are included to modify the taste, smell or appearance (Bisset and Wichtl 2001). It is an established pharmaceutical rule that a herbal mixture should consist of only a few, from four to a maximum of seven, drugs. The licenses for herbal mixtures that are currently prepared, especially in the Germany, specify the limits within which the proportions of the individually active components can be varied within certain limits, but it is also required that the active components make up at least 70% of the mixture (Bisset and Wichtl 2001). In addition to the issues of their activity and desired and undesired side effects, their quality is the most important. The quality assessment of herbal drugs in mixtures has traditionally been based on appearance. An important early visual quality assurance step is to ensure that the plant is of the required species. The medicinal efficacy of many drugs varies greatly between plants of different species of the same genus. The difference can also be extended to different plant parts. Moreover, the different plant part can even represent a different medicinal virtue. Therefore, not only the macroscopic evaluation is needed, drugs should be evaluated microscopically, and phytochemically, as far as possible along the lines of the Pharmacopeias (Bisset and Wichtl 2001). The modern trend towards products in which the native plant structure is destroyed, has eliminated visual assessment from species identification. Such products can range from ground raw plant material to liquid or solid extracts or finished products, including formulations with more than one drug. It then becomes impossible to detect misnaming of species or fraudulent adulteration using macroscopic or organoleptic techniques (Willis et al. 2000). In such cases the only possible identification of species can be achieved by examination of their molecular profiles. TLC is often used as a rapid and not very expensive means of preliminary species identification; some other analytical methods, such as HPLC, MS or GC can be used, but all of them need to be accompanied by a set of standards. Moreover, even a toolbox of modern analytical techniques may not distinguish the compositional variety derived from different species or subspecies from the same genus. One of the up to date methods, DNA fingerprinting with internal transcribed spacers (ITS), offers many advantages, but is currently not used to a great extent (Willis et al. 2000).

The internal transcribed spacers (ITS) region of 18S-28S nuclear ribosomal DNA (nrDNA) has proved to be a useful source for phylogenetic studies (Baldwin et al. 1995). The two spacers of this region, ITS-1 and ITS-2, can be readily amplified and sequenced using universal primers anchored in the conserved 18S and 28S ribosomal RNA genes, even from DNAs from herbarium specimens, if they are preserved from DNA degradation. Furthermore,

the region is non-coding so it evolves rapidly enough to allow most species to be distinguished by sequence variation (Baldwin et al. 1995; Jackson et al. 1999).
In this paper we describe how the ITS region of nrDNA can be used to identify individual species in a sedative tea made of Valerianae radix (*Valeriana officinalis* L.), Lupuli strobuli (*Humulus lupulus* L.), Melissae folium (*Melissa officinalis* L.) and Menthae piperitae folium (*Mentha piperita* L.) using restriction mapping and real-time PCR analysis. According to our knowledge, this is the first identification of drug mixtures by DNA fingerprinting and real-time PCR.

2. Investigations and results

DNA was extracted from the individual drugs and from the tea mixture. Although DNA was recovered in all cases, it was partly degraded (Fig. 1).
ITS sequences of all species were confirmed with sequences retrieved from ITS regions from GeneBank. We supplemented these data by sequencing the complete ITS regions of *V. officinalis* (GeneBank accession number DQ180745) and *M. officinalis* (GeneBank accession number DQ189090).
A DNA sample of *V. officinalis* digested with Nae I produced two fragments of 240 bp and 494 bp; a sample of *H. lupulus* DNA digested with Xcm I, produced two fragments of 261 bp and 469 bp; a DNA sample of *M. officinalis* digested with Nae I and PshA I produced three fragments of 138 bp, 183 bp and 407 bp; a sample of *M. piperita* DNA, digested with Nae I produced two fragments of 137 bp and 595 bp. In all samples of individual herbal drugs primers were also observed (Fig. 2). The restriction profile of sedative tea DNA mixture (Fig. 3) shows the presence of seven fragments. The fragment around 600 bp corresponds to the 595 bp DNA fragment of *M. piperita*; that around 500 bp corresponds to the

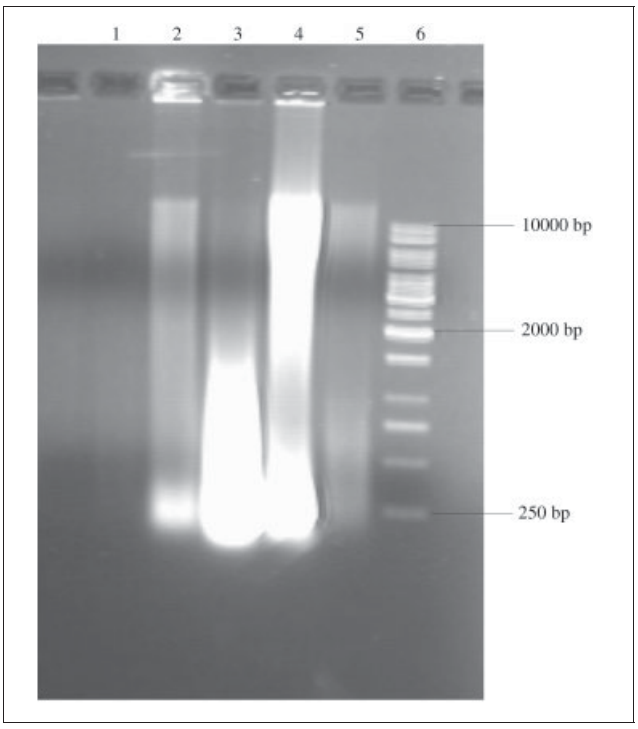

Fig. 1: Genomic DNA isolated from individual herbal drugs and sedative tea. Lane 1 *V. officinalis*; Lane 2 *H. lupulus*; Lane 3 *M. officinalis*; Lane 4 *M. piperita*; Lane 5 sedative tea; Lane 6 Molecular weight markers 1 kpb (GeneRuler, MBI Fermentas)

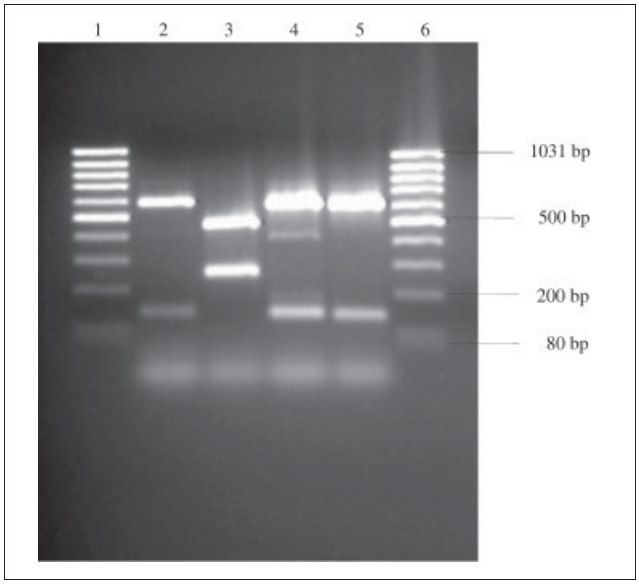

Fig. 2: Restriction analysis of individual herbal drug. Lanes 1 and 6 Molecular weight markers 100 pb (GeneRuler, MBI Fermentas); Lane 2 *V. officinalis*; Lane 3 *H. lupulus*; Lane 4 *M. officinalis*; Lane 5 *M. piperita*

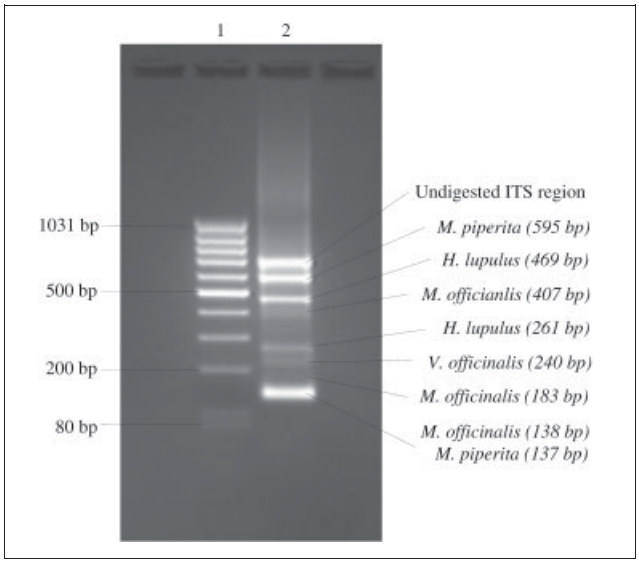

Fig. 3: Restriction profile of tea digestion with Nae I, PshA I and Xcm I. Lane 1 Molecular weight marker 100 pb (GeneRuler, MBI Fermentas); Lane 2 sedative tea

469 bp fragment of *H. lupulus* DNA; that around 400 bp corresponds to *M. officinalis* 407 bp DNA fragment; the one nearest to the 300 bp marker band corresponds to the 261 bp DNA fragment of *H. lupulus*; the next fragment nearest to the 200 bp marked band corresponds to 240 bp DNA fragment of *V. officinalis*; the next DNA fragment corresponds to 183 bp of *M. officinalis* and the smallest fragment corresponds to the DNA fragments of *M. officinalis* (138 bp) and *M. piperita* (137 bp). The observed DNA fragments around 700 bp correspond to the undigested ITS regions when Nae I and PshA I enzymes were used.
To confirm the specificity of the designed primers, the sequences of predicted amplicons were blasted at NCBI. The BLAST search showed a significant degree of identity, E values being $7e^{-58}$, $2e^{-28}$, $4e^{-53}$, $8e^{-38}$ for *V. officinalis*, *H. lupulus*, *M. officinalis* and *M. piperita* ITS 2 regions respectively. Melting curves for each of the following DNA samples: *V. officinalis*, *H. lupulus*, *M. officinalis* and *M.*

piperita showed only one component at 88.5 °C; 79.4 °C; 83.7 °C and 84.6 °C, respectively. No primer dimers were generated during the 45 real-time PCR amplification cycles. We confirmed the presence of all the individual herbal drugs in genomic DNA extracted from the tea mixture. Correlation factors calculated on the basis of linear regressions from relative standard curve for each pair of primers were between 0.9950 and 0.9960, coefficient of variability of Ct values between the triplicates was less the 0,95%. Calculated PCR efficiencies were 1.69, 1.97, 1.86 and 1.98 for samples of *V. officinalis*, *H. lupulus*, *M. officinalis* and *M. piperita* respectively and Fts $3.59E^{-08}$, $6.10E^{-08}$, $6.65E^{-06}$ and $3.46E^{-07}$. Since the Ft of *V. officinalis* was the lowest, all the others Fts including valerianas were divided by it. The values of the initial amounts of genomic DNA for the individual herbal drugs in genomic DNA extracted from tea were in the ratio 1 : 2 : 185 : 10 (*V. officinalis*: *H. lupulus*: *M. officinalis*: *M. piperita*).

3. Discussion

Several methods for isolating the genomic plant DNA were tested. The most undegraded DNA was obtained by using DNeasy Plant Mini Kit (Qiagen, Germany). Even though that DNA was extracted from all the individual herbal drugs as mentioned above it was degraded, the latter was probably due to the high temperature during processing of the herbal drugs and the various storage conditions that result in distortion of the cells, nuclei and genomic DNA. The final concentration of genomic DNA had to be not more then 1.5 ng/μL in order minimise inhibitory influence of polyphenols, polysaccharides and other secondary metabolites that are present in exceptionally high amounts in herbal drugs. It has been reported that, after cell lysis, polyphenols and polysaccharides bind firmly to nucleic acids and in addition to its degradation (John 1992), can also inhibit polymerase or interfere with subsequent reactions (Pirttilä et al. 2001).

Before restriction mapping, sequences were compared to those retrieved from GeneBank, since in some cases there is a variation in ITS sequence within species or even within individuals (Campbell et al. 1997; Kita et al. 2000). Variations were noted and restriction endonucleases were selected in the consensus areas. The digestion buffer was also selected on the basis of the activity of the enzymes. When using a mixture of endonuclease enzymes, a fully optimised reaction buffer can rarely be found; NeBuffer 2 was selected, in which Nae I, PshA I have 75% and Xcm I 100% activity. Despite several attempts incomplete digestion of ITS regions was observed after restriction reaction. Nevertheless this finding did not merely influence the results obtained. Restriction analysis is a relatively fast and qualitative method. However, we found that for restriction profiling of tea mixture we need a comprehensive amount of DNA to enable visualisation of all specific fragments. We therefore decided to use a real-time PCR method, for which the amount of DNA used for amplifying specific regions was 1000 or even 10000 times lower. The calculated ratio of drugs present in the tea mixture did not corresponds to the declared ratio in the tea mixture that is 4 : 3 : 2 : 1 (*V. officinalis*: *H. lupulus*: *M. officinalis*: *M. piperita*) which can be explained by the fact that DNA extraction of an individual herbal drug from the tea mixture is the major limiting step. Different types of plant parts, especially dry express different characteristics so it is almost impossible that the equal amount of DNA could be extracted from leafs compared to the roots. One should also consider the polyploidy of the plants, which can also differ inside the same species as well as between them. Real-time PCR, even though a quantitative method, should be considered in this case only as a qualitative tool that enables specific detection and conformation of species in tea mixtures with a single DNA extraction as well as in a single PCR run. The outcome of the present study may serve as quick and reliable qualitative analytical tool for several pharmaceutical and agricultural needs.

4. Experimental

4.1. Tea and drug samples

A sedative tea mixture made of Valerianae radix, Lupuli strobuli, Melissae folium and Menthae piperitae folium as well as individual herbal drugs from *Valeriana officinalis* L. (Valerianaceae), *Humulus lupulus* L. (Cannabinaceae), *Melissa officinalis* L. (Lamiaceae) and *Mentha piperita* L. (Lamiaceae) were provided by Galex Company, Slovenia. The tea and herbal drugs voucher specimens are deposited at, Faculty of Pharmacy, Department of Pharmaceutical Biology, University of Ljubljana, Slovenia.

4.2. DNA extraction

50–100 mg of tea or individual herbal drug were ground to a fine powder. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacture's protocol. The quality of the DNA was confirmed by 0.8% TAE – agarose gel electrophoresis at 100 V for 1 h and visualized by ethidium bromide (EtBr) staining under UV light (294 nm).

4.3. PCR amplification and sequencing of the ITS region

ITS regions were amplified in 25 μL reaction mixtures composed of 20–50 ng of DNA template, 10 pmol of each primer and 12,5 μL PCR-Master Mix (Promega, USA). The primers ITS_F (5′AGAAAGTCGTAAGTCGTAACAAGGTTTCCGTAG3′) and ITS_R (5′TTTTCCTCCGCTCATTGATATGCTT3′) were based on *Arabidopsis thaliana* nrDNA sequences. Amplification was carried out in a Primus 96 Plus Cycler (MWG Biotech, Germany). The PCR program included predenaturation for 1 min at 96 °C, 35 cycles of 0.5 min at 96 °C, 0.5 min at 55 °C, and 0.5 min at 72 °C, followed by a single cycle of 1 min elongation at 72 °C. Fragments were subcloned in pGEM T Easy vector and sequenced using universal primers SP6 primer and T7 promotor primer by MWG Biotech, Germany. Use of both primers enabled further confirmation of the sequences of the target DNA fragments. Sequences were verified using BLAST protocol at the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST/).

4.4. Restriction analysis

Appropriate restriction enzymes were identified on the basis of alignment of sequences obtained from sequencing and ITS sequences retrieved from GeneBank of the species under consideration, using WebCutter (WebCutter 2.0, http://www.firstmarket.com/cutter/cut2.html). According to the restriction map, restriction enzymes Nae I, PshA I, and Xcm I (New England BioLabs, England) were selected as suitable candidates for determining the different drugs present in the tea mixture. The appropriateness of the candidate enzymes was confirmed by digestion of ITS regions of individual species. The use of the restriction fragments to identify the tea mixture was tested by amplifying the ITS regions from genomic DNA isolated from the tea mixture and digestion with a mixture of selected restriction enzymes. Digestion was performed in NeBuffer 2 buffer according to the manufacturer's protocol. Restriction fragments were separated by 2% TAE – agarose gel electrophoresis at 100 V for 1 h and visualized by EtBr staining under UV light (294 nm).

4.5. Real-time polymerase chain reaction (SYBR Green assay)

Specific primers based on ITS sequences specific for individual species as templates and the computer software Primer Express® (Applied Biosystems) with default parameter and criteria settings. *V. officinalis*: V_F 5′GCTGGCCTAAAACACGG3′, V_R: 5′GTGGACGGCACGTATGG3′; *H. lupulus*: L_F 5′CCGTTGCCCCCCTTGA3′, L_R 5′CCAATCTCCCCCCTCCCCCCACTT3′; *M. officinalis*: Mel_F 5′ATCCCTCGGCGACTGGCGACTCATG3′, Mel_R 5′TGTGAGGCACCGACACCAT3′; *M. piperita*: M_F 5′TGGTGGTTGAACATCTCAATCTCT3′, M_R 5′CACCGTTGGGTCGTTTGTG3′. Sequences were blasted for specificity and discrimination with BLAST protocol at NCBI.

The PCR reactions were carried out in 25 μL reactions mixtures composed of 12.5 μL Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogene, Life technologies), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer

(10 μM), 0.5 μL ROX Reference Dye, 6 μL autoclaved distilled water and 5 μL DNA template or distilled water as non-template control. For signal detection an ABI Prism 7000 (Applied Biosystems, Switzerland) was programmed to an initial step of 2 min at 50 °C and 2 min at 95 °C, followed by 45 thermal cycles of 0.2 min at 95 °C, 0.5 min at 55 °C and 0.5 min at 72 °C. Each sample was tested in triplicate. Threshold cycles and baseline were determined automatically by SDS software also provided by Applied Biosystems. Primer specificity was confirmed by DNA melting curves and their first derivative melting peaks, using the software for automatic capture fluorescence data during a gradual temperature increase from 60 to 95 °C. Specific amplicons were also amplified using DNA from PCR product amplified with ITS primers, individual genomic DNA and, individual ITS regions subcloned in pGEM T Easy vector as positive controls.

A relative standard curve for each herbal drug was generated by plotting the logarithm of serial dilutions of genomic DNA from the tea mixture against the average Ct value for each dilution. Slope and y-intercept of standard curve line were calculated by linear regression. PCR efficiency was calculated according to the equation $\mathrm{Eff} = 10^{(-1/\mathrm{slope})}$. A threshold factor (Ft) of threshold amount of amplicons is defined according to equation $F_t = \mathrm{Eff}^{-\mathrm{Ct}}$. Ratio between initial amounts of genomic DNA for individual herbal drug in genomic DNA extracted from tea was calculated by dividing the Ft for each drug by the minimum Ft calculated.

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