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Determination of shelf life of *Chelidonium majus*, *Sambucus nigra*, *Thymus vulgaris* and *Thymus serpyllum* herbal tinctures by various stability-indicating tests

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ABSTRACT

Stability testing of herbal preparations has recently been recognized as essential for quality control to support their shelf life. Various stability-indicating tests were assessed for their predictive power in herbal tincture stability testing and compared to reference quantitative determination of marker compounds. Herbs from Western herbal medicine with different active constituents were selected: *Chelidonium majus*, *Sambucus nigra* flowers, *Thymus serpyllum* and *Thymus vulgaris*. Their freshly prepared and commercially available tinctures were tested for stability under normal and accelerated conditions. Quantitative chromatographic assays were developed for chelidonine in *Chelidonium* tincture, for isoquercitrin, rutin and quercetin in *Sambucus* tinctures, and for thymol in *Thymus* tinctures. Additional procedures were assessed for their predictive power on tincture stability: chromatographic profiling, spectrophotometric evaluation of tincture colour, DPPH antioxidant assay. With the exception of the DPPH assay in *Sambucus* tincture, none of the assessed stability-indicating tests was satisfactory in comparison with reference determination. *Sambucus* tincture was stable for more than six months, while *Chelidonium* and *Thymus* tinctures were stable for less than 1.5 months with the decrease in marker compound >10%. Compound borneol is proposed as a marker for the deterioration of *Thymus* tinctures. *Chelidonium* tincture was additionally tested for its stability on UV and visible light. Chelidonine degrades in the tincture under the visible light. The results of the present study implicate that the only assays suitable for stability testing of different tinctures are those that determine concentration of the active compounds or some active compound-linked property of the tincture.

1. Introduction

Stability testing for active substances is an important and mandatory aspect of quality assurance in the pharmaceutical industry, either in their pure form or in formulations. Protocols for stability testing are prescribed by an International Conference on Harmonization (ICH) in ICH Topic Q1A Stability Testing Guidelines (Anon, CPMP/ICH/380/95, 1998). For herbal preparations, stability testing has come under the spotlight only recently due to increasing evidence of public health risk from low grade herbal products. The Traditional Herbal Medicines Directive (Anon, DIRECTIVE 2004/24/EC, 2004) required each EU member state to put in a registration scheme for manufactured traditional herbal medicines. Application for a traditional use registration requires the applicant to compile a dossier broadly covering the proof of traditional use, safety and quality of which stability testing is a recent yet essential requisite. The guidelines for stability testing have been specifically adapted by European Medicines Agency – EMEA, Committee for Herbal Medicinal Products – HMPC (Anon, CPMP/QWP/2820/00 Rev 1, 2006). Compared with pharmaceutical active compounds, there are important differences in the proposed procedures. Either constituents with known therapeutic activity or markers should be quantified during the stability testing, which should last at least six months and the limit is set to ±5% of the initial value in the case of known active constituent and ±10% of the initial value for the marker compound. However, an additional condition should be met: design and implementation of additional product-specific stability-indicating tests. They should be appropriate for detecting any changes in the product quality during its shelf-life (Anon, CPMP/QWP/2820/00 Rev 1, 2006).

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Currently, stability testing of herbal tinctures and other preparations is done predominantly by chromatographic fingerprinting and quantitative determination of active or marker constituents (Bilia et al., 2001, 2006, 2007, 2002a,b; Bos et al., 1996). The possible drawback is the need to extract samples in order to minimize the influence of the interferences on determination. Various detectors are used, but by far the most specific is the mass spectrometer. In order to avoid the loss of compounds in the sample preparation and chromatographic separation steps, more elaborate combination of mass spectrometry and nuclear magnetic resonance has been proposed for tincture testing, but it involves the use of elaborate and expensive equipment (Politi et al., 2009). Another interesting approach to stability testing is the application of the appropriate biochemical assays, e.g. in vitro immune assay to test the stability of *Echinacea* spp. preparations (Senchina et al., 2005).

The tinctures of commonly used Europaean medicinal plants (greater celandine – *Chelidonium majus* L., Papaveraceae; garden thyme – *Thymus vulgaris* L. and wild thyme – *Thymus serpyllum* L., both Lamiaceae; elder – *Sambucus nigra* L., Adoxaceae) were chosen for the testing on the basis of chemically different active constituents, with the aim of evaluating the stability testing protocols for diverse possible herbal constituents. The chemical profiles of these medicinal plants have already been extensively studied. To the best of our efforts, we have not found any studies regarding the stability testing of the tinctures made from these herbs. Kaack and Christensen (2010) followed the content of various phenolic compounds in dried elderflower herb packed in different materials over a period of 21 months. They concluded that phenolic compounds were stable under the applied storage conditions.

The active constituents of the greater celandine (*Chelidonium majus*) are most likely its alkaloids (El-Readi et al., 2013; Ernst and Schmidt, 2005; Gilca et al., 2010; Monavari et al., 2012; Yang et al., 2011). The plant is traditionally used in Western herbal medicine internally as a choleretic, cholagogue and hepatoprotective and externally as an antifungal and antiviral, specifically for common warts (verrucae) (Gilca et al., 2010; Monavari et al., 2012). Research has shown the effectiveness of its alkaloids against MRSA and *Mycobacterium tuberculosis* (Liang et al., 2011; Zuo et al., 2011) and against cancer cells (El-Readi et al., 2013). An effective anti-cancer drug Ukrain has been developed from its extract (Ernst and Schmidt, 2005). In animal models, it has also shown promising results in the treatment of atopic dermatitis (Yang et al., 2011). Other isolated compounds have shown interesting properties as well, e.g. a cysteine proteinase inhibitor might be responsible for the antiviral activity of the sap (Rogelj et al., 1988), and chelidonic acid has shown anti-inflammatory properties (Shin et al., 2011), useful in the treatment of ulcerative colitis (Kim et al., 2012).

Both garden and wild thyme (*Thymus vulgaris* and *Th. serpyllum*, respectively) are traditionally used as expectorants and antimicrobials in upper respiratory tract infections because of their bronchoantispasmodic, expectorant and antibacterial activity (Blumenthal et al., 2000; Staszek et al., 2014). Antibacterial activity is attributed to its essential oil (Lakis et al., 2012; Sfeir et al., 2013), containing phenolic compounds thymol and carvacrol (Móricz et al., 2012; Staszek et al., 2014). Spasmolytic effect is attributed to the same, in synergy with the flavonoids (Engelbertz et al., 2012). In addition to the effect on the respiratory system, thyme preparations have also been shown to be effective spasmolytics in dysmenorrhaea (Direkvand-Moghadam and Khosravi, 2012). Many species of *Thymus* are used in the traditional medicine for the same purpose. Additionally, same species of thyme can show wide phenotypic variation, resulting in chemical polymorphism and different ratio of constituents (De Lisi et al., 2011; Staszek et al., 2014; Thompson et al., 2013). In spite of that, thymol and carvacrol have been proposed as markers for the authentication and quality control of thyme species (Sgorbini et al., 2015).

In elder (*Sambucus nigra*), both flowers and berries are used in the traditional medicine. Flowers (Sambuci flos) are used in colds (Blumenthal et al., 2000), in feverish conditions and as an anticatarrhal in allergic conditions. The active constituents are presumably hydroxycinnamic acids and flavonol glycosides (Dawidowicz et al., 2006; Mikulic-Petkovsek et al., 2015). One of the most abundant glycosides, isoquercitrin, has been shown to inhibit histamine release from mast cells (Matsumoto et al., 2009) and to exert an anti-inflammatory effect in an asthma model (Rogerio et al., 2007). Recently, acyl spermidines have been identified as constituents of elderflowers, possibly contributing to their cold-alleviating properties (Kite et al., 2013). Herb quality is assessed by determination of flavonoid content, calculated as isoquercitrin (Blumenthal et al., 2000).

In the present work, we aimed to assess the stability of freshly prepared and commercially available tinctures by comparing the established approach, i.e. monitoring of the active or marker constituents with the appropriate chromatographic method, with more simple procedures as stability-indicating tests, thus meeting the EMEA requirements of additional testing (Anon, CPMP/QWP/2820/00 Rev 1, 2006). The procedures should be simple in order to enable their rapid application and interpretation. Monitoring of colour change by spectrophotometric means and an antioxidant test with a stable DPPH radical (Brand-Williams et al., 1995) were evaluated for their predictive power in the stability testing. They were compared with the quantitative determination of at least one of the possible active constituents in each herb: alkaloid chelidonine in *Chelidonium majus*; phenolic compound thymol in *Thymus* sp.; flavonoids isoquercitrin, rutin and quercetin in *Sambucus nigra* flowers (Fig. 1).

2. Experimental

2.1. Chemicals and materials

The following standard compounds were used: chelidonine (Sigma, USA, >97%); thymol (Fluka, Germany, >99%); quercetin (Sigma, USA, anhydrous); isoquercitrin (Fluka, Germany, >90%); rutin (Sigma, USA, >94%). Stable DPPH (2,2-diphenyl-picrylhydrazyl) radical was purchased from Sigma-Aldrich, USA. Solvents methanol, acetonitrile, *n*-propanol were HPLC grade from Sigma, USA. Deionised water was prepared by MilliQ system (Millipore Corp., USA). Other chemicals were *p.a.* grade from various producers.

Stock solutions of active compounds were prepared in methanol or *n*-propanol by dissolving accurately weighed solid standard to give a concentration of 0.5–1.0 mg/ml. They were stored in refrigerator and stable for several months. Calibration solutions were prepared by dilution of stock solutions in deionised water to the desired concentration as specified under individual methods.

Plant materials were collected in their appropriate season (greater celandine whole plant in July 2011; thyme aerial parts in June 2011; elderflowers in June 2011) in the south-western part of Slovenia. They were identified by the authors, who are trained medical herbalists. No voucher specimens were retained. Collected plants were dried and stored in paper bags in a cool, dark place, and were subjected to tincture preparation immediately before the beginning of the stability testing. Tinctures were prepared at the latest date six months after the collection of herbs by the cold percolation method in the mixture of tap water and food-grade ethanol in the same strength and solvent ratios as those used for commercial manufacture: *Chelidonium majus* 1:5 (45% ethanol);