ASSESSMENT OF THE GRANULATION PROCESS EFFECT ON MORPHO-ANATOMICAL FEATURES AND BIOLOGICALLY ACTIVE SUBSTANCES COMPOSITION OF SENNA LEAVES

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Abstract

Cassia senna L. and Cassia angustifolia Vahl. leaves and their preparations are widely acknowledged herbal drugs. Recent development of a novel dosage form - cut-pressed granules - enhances convenience of use in a home setting. However, manufacturing of cut-pressed granules is feasible only if the quality of the material doesn’t decrease during granulation. The aim of the study was to assess the possible changes in identification parameters and in the composition of biologically active substances in Senna leaves after processing. It was shown that TLC chromatographic profiles of the herbal drug remain unchanged. Spectrophotometric assessment confirmed that the anthracene aglycones content of the herbal material was also not affected by granulation. It can be concluded that the quality of cut-pressed granules obtained from Senna leaves is not inferior compared to the raw material.

Keywords: herbal raw material, dosage form, cut-pressed granules, Senna leaves

Introduction

Medicinal plants were used for health benefits since the dawn of humankind. To this day, they remain an important part of the healthcare all over the world. It is estimated that every 3rd medicinal product on the world market is of plant origin. In the Russian Federation, as well as in Japan and South-eastern Asia countries, herbal drug products comprise about 40% of the overall number of marketed medicines used [15].

Senna infusions act as a laxative due to chemical irritation of bowel mucosa, which results in enhanced peristaltic contractions. These preparations are prescribed to promote regular bowel movements in patients with haemorrhoids, non-acute proctitis, and anal fissures, and in those with chronic constipation due to hypo- or atonicity of large intestine [28]. Recent advances in pharmacological studies of Senna species showed that crude extracts of *C. senna* demonstrate in vitro activity against Gram-negative bacteria [11]. Methanolic fraction of *C. angustifolia* leaf extract showed hepatoprotective effect in a rat model of carbon tetrachloride-induced hepatic toxicity, significantly reducing levels...
of hepatic markers and preventing hepatic lesions formation [3]. Ethanol extract of *C. senna* L. was able to suppress malignant proliferation of prostate ductal epithelial cells *in vivo*, thus hinting its possible anticancer activity [13].

Currently, leaves of both *C. angustifolia* Vahl. and *C. senna* L. are recognized within single pharmacopoeial monograph, i.e., either species (or their mixture) can be used [25, 27]. Despite some minor differences in botanical description and chemical constituents, both species contain sennosides A, B, C and D - glycosides, responsible for pharmacological activity of *Senna* [7]. Recently, it was shown that *C. angustifolia* Vahl. contains several flavonoids, namely quercimeritin, scutellarein, and rutin, that show anticancer, anti-oxidant, and antimicrobial properties [1].

Novel identification methods for pharmacopoeial Senna species include high-performance thin-layer chromatography [14], as well as scanning electron microscopy method for authentication of *C. angustifolia* Vahl. [20]. However, current pharmacopoeial method for identification of both *C. senna* L. and *C. angustifolia* Vahl. still relies on time-proven, classic light microscopy technique [25-27].

Herbal drug products are manufactured using either whole, cut, or powdered herbal raw material and are usually released in packs or in tea filter bags. *Senna* leaves in filter bags, which contain coarse herbal powder in quantities necessary for a single dose of aqueous extract, are convenient for use. However, both cut and powdered herbal raw material is difficult to process and package, as the irregular particles of the material demonstrate fluctuating physical characteristics that depend on the type and the part of the plant being processed. Introduction of novel dosage form - cut-pressed granules - improves technological parameters of *Senna* leaves preparation (flowability, homogeneity, uniformity of packaging); thus, the product range of herbal medicines is further expanded [6-7, 19, 21-23]. However, the process includes treatment of herbal raw material with saturated steam and consequent drying, which may negatively influence quality (i.e., alter composition of biologically active substance and morpho-anatomical features) of *Senna* leaves. It is widely accepted that temperature and relative humidity (RH) are the primary parameters that influence stability of active pharmaceutical ingredients, excipients [4], and dosage forms [24]. Both of the factors demonstrate combined contribution to the chemical degradation processes; e.g., the rate of hydrolysis may be increased both by moisture content and temperature.

Despite being rarely tested for stability, herbal drugs and herbal raw materials are also prone to degradation under stress conditions [2]. For example, it was shown that no significant changes in chemical composition of commercially available *Senna* leaves was observed at 25°C/60% RH. However, even the small increase in temperature and relative humidity (up to 40°C/75% RH) demonstrated rapid degradation of all major constituents, among them rhein-8-O-glucoside, sennosides and flavonoids [8]. Other *Cassia* species, such as *Cassia alata* L., may lose their biological activity at increased temperatures due to changes in concentration of active moieties [17-18].

Taking this into account, the aim of our work was to assess changes in identification parameters and in composition of biologically active substances of *Senna* (*Cassia senna* L. and *Cassia angustifolia* Vahl.) leaves that may arise during manufacturing of *Senna* cut-pressed granules.

**Materials and Methods**

*Plant material.* Commercial batches of cut *Senna* leaves (mixture of *Cassia acutifolia* Del. (C. *Senna* L.) and *Cassia angustifolia* Vahl.) corresponding to the requirements of the Russian State Pharmacopoeia, monograph 2.5.0038.15 “*Senna* leaves” were used in the study [27].

In order to obtain cut-pressed granules, powdered *Senna* leaves (particle size NMT 2 mm) were evenly moistened using saturated steam (time – 3 - 4 minutes, vapour pressure – 3.5 - 5.5 kgf/cm²) under constant stirring. Then the material was transferred to a compression machine in which the moistened mass was pushed through a 5 - 7 mm sieve. The material was extruded from the machine in form of 10 - 30 mm cylinders, which were transferred to the dryer. After drying, the material was force-cooled with air and was transferred to a roll grinder in which it was crushed to granules passing through a 2 mm sieve.

*TLC identification.* Qualitative analysis of phenolic compounds in *Senna* leaves preparations (powder and cut-pressed granules obtained from mixture of *C. senna* L. and *C. angustifolia* Vahl.) was performed using thin-layer chromatography (TLC) on TLC Silica gel 60 F254 plates (Merck, Germany).

Samples of herbal raw material and granules were powdered, obtaining particles about 1 mm in size. About 1.0 g of the powdered raw material or granules were placed into a 100 mL conical flask with ground glass joint, 25 mL of 96% alcohol and water mixture (1:1) were added, and the flask was heated under backflow condenser on a boiling water bath for 5 minutes. After cooling, the extract was filtered through a paper filter (Test solution).

**Sennoside B reference standard (RS) solution** was prepared by weighing about 0.001 g of sennoside B RS (purity ≥ 90%), dissolving it in 5 mL of 96% alcohol and water mixture (1:1) and mixing. **Barbaloin RS solution** was prepared by weighing about 0.002 g of barbaloin (aloin) RS (purity ≥ 95%), dissolving it in 1 mL of 70% alcohol, and mixing. Test solution (10 μL), Sennoside B RS solution (10 μL), and Barbaloin RS solution (5 μL) were applied to the start line of the 100 mm × 100 mm chromatographic
plate as 10 mm bands. The plates were dried for 15 min at room temperature, then transferred to the chromatographic chambers (Camag, Switzerland), previously lined with filter paper and saturated for 60 min with a mixture of solvents (glacial acetic acid/water/ethyl acetate/propanol (0.5:10:20:20)), and chromatographed using the same system. Obtained chromatograms were sprayed with 20% nitric acid solution and 5% potassium hydroxide alcoholic solution.

Photographs of the chromatograms were taken using Reprostat 3 TLC imaging system (Camag, Switzerland) and then processed using Adobe Photoshop 7.0 (Adobe, USA).

**Anthracene aglycones assay.** The effect of granulation process on anthracene aglycones content (expressed as chrysophanic acid) was assessed using UV/VIS-spectrophotometry. The assay was performed in triplicate.

Samples of herbal raw material and granules (obtained from mixture of *C. senna* L. and *C. angustifolia* Vahl) were powdered, obtaining particles about 1 mm in size. About 0.4 g of the powdered raw material or granules were accurately weighed into a 250 mL flask with ground glass joint and 100 mL of purified water were added. The content was mixed for 10 min, then heated under backflow condenser in a boiling water bath (water should be at the same level as the liquid in the flask) for 20 min, stirring the content periodically. After cooling under a stream of water, the flask was left for 10 min at room temperature, and then its content was filtered through a folded paper filter.

Next, 25 mL of the filtrate were transferred into a 100 mL separatory funnel, and the extraction was performed twice, using 40 mL and 20 mL of ether. After extraction, the filtrate was transferred into a 250 mL flask with ground glass joint. Combined ether extracts were washed twice, using 10 mL of purified water per washing. The water was then separated and combined with the filtrate in the 250 mL flask. Ether extracts were discarded.

The flask with water extracts was heated on a water bath until ether smell disappeared. Then, 0.1 g of sodium hydrogen carbonate and 10 mL of iron (III) chloride (density 1.07 - 1.08) were added to the flask, it was connected to a backflow condenser, and the mixture was heated in a boiling water bath for 20 min, stirring periodically. After 20 min, 5 mL of 50% sulfuric acid solution were added to the mixture, and the heating continued for 30 more min. After cooling, the solution was transferred to a 300 mL separatory funnel, and the flask was rinsed consequentially with 20 mL of purified water and 75 mL of ether. The washings and ether were added to the solution in the funnel and its content was shaken for 5 min. After separation, ether layer was transferred into a 500 mL separatory funnel, leaving dark floccules in the aqueous layer. The extraction from the aqueous layer was repeated twice, using 30 mL and 20 mL portions of ether. Combined ether extracts were filtered through a glass filter (P 100), and then washed twice with 30 mL portions of water. 100 mL of alkaline ammonium solution (50 g of sodium hydroxide were dissolved under stirring in 870 mL of purified water, then, after cooling, 80 mL of concentrated 25% ammonia solution were added) were added to the ether extract and mixed. Obtained mixture was carefully shaken for 5 min. After separation, transparent aqueous layer was decanted into a 250 mL volumetric flask, making sure that the floccules remain in the funnel. Then, 20 mL of purified water and 3 mL of concentrated hydrochloric acid were added to the ether extract, the funnel was cooled under stream of water, and shaken for 2 min. Following separation, aqueous layer was decanted into the same volumetric flask.

Ether extract was shaken with another 50 mL portion of alkaline ammonium solution for 2 min, and, after separation, the aqueous layer was decanted into the same volumetric flask. The content of the flask was diluted to volume with alkaline ammonium solution and mixed. After 15 min, the absorbance of the solution was measured at 523 nm in a 10 mm cuvette, using alkaline ammonium solution as a blank.

Total anthracene aglycones content (expressed as percent (X) of chrysophanic acid per g of dry material) was calculated using the following formula:

\[ X = \frac{A \times 100000}{432 \times a \times (100 - W)} \]

where: \( A \) – absorbance of the solution; \( 432 \) – specific absorbance of chrysophanic acid at 523 nm; \( a \) – mass of the analysed sample, g; \( W \) – moisture content, %.

**Morpho-anatomical assessment.** In order to assess possible changes in morpho-anatomical features of the studied herbal material, powdered Senna leaves and cut-pressed granules were used to obtain slides for microscopic examination [27]. About 0.1 g of either powder or powdered granules were placed into 50 mL beaker, 5 - 10 mL of 5% sodium hydroxide solution were added, and the content was boiled for about five minutes. After that the powder was subjected to fractional washing using 100 - 150 mL portions of purified water that was decanted after complete sedimentation of the particles. Following last decantation the powder was transferred into a drop of inclusive fluid (glycerol water solution (1:1)) using spatula, covered with a cover glass, slightly pressed by the reverse side of a dissecting needle and examined under a microscope.

Photographs were taken using Olympus CX41 (Olympus, Japan) microscope equipped with x10 eyepiece, x4, x20 and x40 lenses, and a digital camera PowerShot G1X (Canon, Japan). All photographs were processed using Adobe Photoshop CS 6 (Adobe, USA).
Results and Discussion

Currently, several extraction and preparation methods are employed for obtaining herbal drugs from herbal raw materials, the most widely used being hydrodistillation extraction and accelerated solvent extraction (ASE). However, since they rely on elevated temperatures and/or humidity in order to increase yield of biologically active substances from the source material, manufacturing process conditions must be strictly controlled in order to prevent thermal degradation and hydrolysis [16]. Absence of material degradation, and, therefore, absence of drug product quality decrease, should be confirmed qualitatively and quantitatively.

**TLC identification:** After solvent front has passed about 80 - 90% of the TLC plate length from the start line, the plate was removed from the chamber and air-dried until full evaporation of solvent residues. The chromatograms were sprayed with 20% nitric acid solution and heated in a temperature chamber at 100 - 105ºС for 10 min, then cooled to the room temperature, sprayed with 5% alcoholic solution of potassium hydroxide and air-dried under fume hood. TLC plates were examined under daylight.

The chromatogram of Sennoside B RS solution and Barbaloine RS solution showed brown-violet or gray-violet zone (sennoside B) and violet-brown or brown zone above it (barbaloine).

The chromatograms of all Test solutions showed brown-violet or gray-violet zone with the same Rf as the sennoside B, and red-brown or brown zone with the same Rf as the barbaloin. Between these two zones, two brown-violet or gray-violet zones, and one red-brown or brown zone can be observed. Other zones may be present.

Chromatographic profiles of powdered Senna leaves and pilot samples of cut-pressed granules were found to be similar (Figure 1).

Current pharmacopoeial approaches to Senna herbal raw material and herbal drug products identification employ TLC, despite minor differences. In a classic work by Khafagy et al. an isopropanol/ethyl acetate/water (36:36:28) developing system is described [12]. Both Russian State Pharmacopoeia and European Pharmacopoeia 10th ed. utilize TLC mobile phases consisting of glacial acetic acid/water/ethyl acetate/propanol in slightly different ratios – 0.5:10:20:20 and 1:30:40:40, respectively [25, 27]. However, mobile phase described in Chinese Pharmacopoeia somewhat differs: its comprised of ethyl acetate/propanol/water (4:4:3) [26]. Such disagreement can be explained by the fact that Russian State Pharmacopoeia employs reference standards as markers, rather than reference drug solution or Senna extract certified reference standard. It should be noted that instead of sennosides A and B (main active constituents of Senna leaves, dianthrone O-glycosides) mixture, we utilized mixture of sennoside B and barbaloin reference standards. This is justified by the necessity to demonstrate stability of minor constituents, as the cut-pressed granules manufacturing process may also affect their composition, thus distorting TLC profile.

Notwithstanding mentioned differences, all of the pharmacopoeial sources agree that the spots in the...
chromatograms of test solution and reference should match in position and colour. Similarity of chromatographic profiles obtained for Senna herbal raw material and cut-pressed granules allow us to conclude that the granulation process did not affect major and minor chemical constituents of Senna leaves.

**Anthracene aglycones assay:**
Current pharmacopoeial assay for Senna leaves is based on spectrophotometric method [25, 27]. According to the European Pharmacopoeia, Senna leaves should contain not less than 2.5% of hydroxyanthracene glycosides, expressed as sennoside B, as major pharmacological activity of Senna leaves is due to anthracene glycosides rather than anthracene aglycones [9]. However, Senna monograph of the Russian State Pharmacopoeia describes different requirements: not less than 1.35% of anthracene aglycones, expressed as chrysophanic acid. This is justified by the necessity to take all anthracene derivatives into account as they demonstrate variety of other pharmacological activities [10].

The results (Table I) of the cut-pressed granules spectrophotometric analysis showed that total anthracene aglycones content remained within specified limits (NLT 1.35%) [27].

| Batch No. | Total anthracene aglycones content (expressed as chrysophanic acid), % | Sample No. | Total anthracene aglycones content (expressed as chrysophanic acid), % |
|-----------|--------------------------------------------------------------------------------|-------------|--------------------------------------------------------------------------------|
| 221115    | 1.39 | Cut-pressed granules from batch 221115                          | 1.38 |
| 70416     | 1.40 | Cut-pressed granules from batch 70416                          | 1.40 |
| 160617    | 1.36 | Cut-pressed granules from batch 160617                          | 1.37 |
| 100418    | 1.39 | Cut-pressed granules from batch 100418                          | 1.41 |

From the assay results it can be concluded that the granulation process had no negative effect on quantitative composition of biologically active substances of senna leaves.

**Morpho-anatomical assessment:**
High processing temperatures and/or humidity can alter morphological characteristics of herbal raw material, hindering identification of herbal drug products. Identification of herbal drugs and raw material is usually performed using light microscopy, as the method is well-established, described in the literature, and adopted by pharmacopoeias [20, 25-27]. All sources describe similar characteristics for Senna leaves. Microscopic examination of Senna powder and cut-pressed granules revealed fragments, characteristic for the herbal raw material (Table II).

**Table II**

Comparison of morpho-anatomical features of the powder and cut-pressed granules obtained from Senna leaves (×200)

Leaf epidermis fragment with polygonal epidermal cells, paracytic stomata, unicellular conical trichomes with warded walls.
Based on similarity of morpho-anatomical features between Senna raw material and cut-pressed granules it can be concluded the granulation process has no effect on characteristics, essential for microscopic identification.

Conclusions
Using commonly accepted analytical techniques (microscopic identification, TLC fingerprinting, and spectrophotometric assay) we were able to confirm that treatment of Senna (*Cassia senna* L. and *Cassia angustifolia* Vahl.) leaves during manufacturing of cut-pressed granules does not alter their identification parameters and content of biologically active compounds.

Conflict of interest
The authors declare no conflict of interest.

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