STANDARDIZATION OF CHAGA TINCTURE AND BEFUNGIN

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Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 44, No. 3, pp. 35 – 37, March, 2010.

Original article submitted June 18, 2009.

Methods for standardizing the formulations Chaga Tincture and Befungin were developed, consisting of extraction of the non-phenol fraction predominantly containing tetracyclic triterpenes, including lanosterol and ergosterol, followed by spectrophotometry of colored complexes with vanillin in acidic conditions. The contents of tetracyclic triterpenes expressed as lanosterol ranged from 0.01% to 0.035% in Chaga Tincture and from 0.01% to 0.02% in Befungin.

Key words: Chaga, standardization, colored complex, spectrophotometry, non-phenol fraction, tetracyclic triterpenes, lanosterol.

Chaga is the shelf fungus *Inonotus obliquus* (Pers.) Pil., family *Hymenochaetaceae*, which has long been used in folk and scientific medicine. Chaga preparations have reinforcing, tonic, and antitumor activities and are used in the treatment and prophylaxis of gastrointestinal diseases, in the post-operative treatment of malignant tumors, and in chronic fatigue syndrome [1, 2].

Biologically active additives based on chaga extracts are currently used in Russia: Chagovit, available as capsules and an elixir, Éktrabesungin, Berezka balsam, Litovit Ch tablets, dry chaga extract, and cryoprepared powder. There is significant demand for the medicinal formulations Chaga Tincture and Befungin. Lack of supply of these formulations arises predominantly from the fact that producers experience difficulties associated with standardization. The Normative Documents for Befungin (Pharmacopeia Monograph FS 42-3991-83), Chaga Tincture (FS 42-659-94), and Chaga (State Pharmacopeia, 11th Edition, Part 2, p. 63) are severely out of date and to not correspond to current requirements. These articles provide qualitative and quantitative determinations of a "Chromogenic complex" – a vague concept with no definition of its chemical composition, based on gravimetric measurement.

Chaga as an object of chemical studies contains a complex of biologically active compounds of different classes, such as phenols (including polyphenols), aliphatic acids (oxalic, acetic, formic), and aromatic acids (hydroxybenzoic and vanillic acids), coumarins (peucidanin), lipids, tetracyclic triterpenes (inonotic acid, obliquic acid, inotodiol, lanosterol), and steroids (ergosterol). Chaga contains trace elements in the form of oxides of copper, barium, magnesium, aluminum, sodium, and calcium [1 – 5].

The marked pharmacological activities of chaga and preparations made from it result from the presence of different classes of biologically active substances and have stimulated chemical studies of its composition. Attempts have been made to standardize chaga preparations and raw material using contemporary approaches. Some studies [6, 7] have shown that Befungin can be standardized by spectrophotometry of colored complexes of free carbohydrate and free phenols present in the formulation. The Pharmacopoeia monograph is based on the assay of free phenols in the chloroform fraction. A significant disadvantage of these methods is that standardization of the formulation is based on substances which are not responsible for the pharmacological activity of Befungin. Extraction of free phenol fractions is accompanied by the formation of a stable emulsion, which not only hinders the assay, but also significantly distorts the results.

Thus, these points identify a need to develop methods for the quantitative and qualitative analysis of Befungin, Chaga Tincture, and the raw material. We were guided by the view that standardization of formulations should be based on the class of biologically active compounds responsible for their pharmacological activity. Data in the literature available to us indicate that the biological activity of chaga extracts is associated with triterpenes: inonotic acid, obliquic acid, inotodiol, and lanosterol. Inotodiol and lanosterol have antiblastic (antitumor) activity [1, 8, 9].

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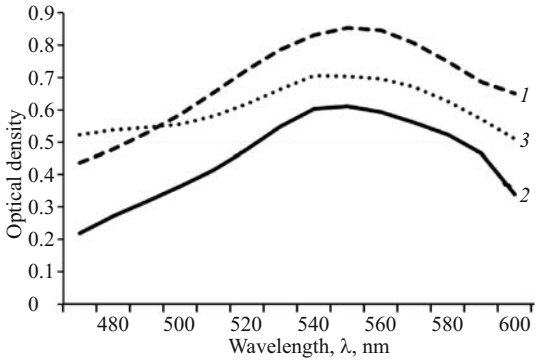

Fig. 1. UV spectra of colored complexes of lanosterol, Chaga Tincture, and Befungin: *1*) Chaga Tincture; *2*) lanosterol; *3*) Befungin.

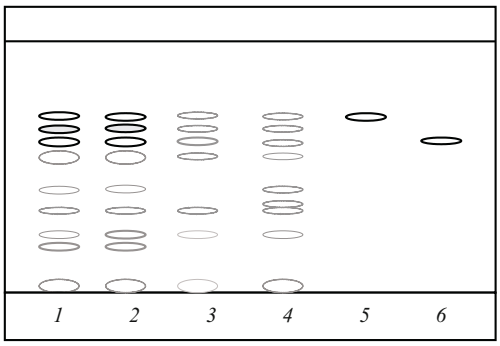

Fig. 2. Diagram of chromatogram of tetracyclic triterpene fractions purified from raw chaga material and extracts in a chloroform-methanol (25:1) solvent system: *1*) Chaga Tincture; *2*) raw chaga material (extracted); *3*) Befungin; *4*) propylene glycol extract; *5*) lanosterol; *6*) ergosterol.

Our studies led to the extraction of non-phenol fractions from formulations and raw material, these fractions predominantly containing tetracyclic triterpenes and steroids, including lanosterol and ergosterol. A further objective was to obtain a stable colored complex of tetracyclic triterpenes with vanillin in acidic conditions, followed by spectrophotometry, using the colored complex of a lanosterol standard as the reference compound. Methods developed on the basis of the non-phenol fraction of tetracyclic triterpenes should provide good qualitative and quantitative characterization not only of Chaga Tincture, Befungin, and water-ethanol and propylene glycol extracts produced by OOO Kameliya NPP, but also the raw material, i.e., chaga. We describe here a method for the qualitative and quantitative estimation of the total content of tetracyclic triterpenes (expressed as lanosterol) using Chaga Tincture as an example.

EXPERIMENTAL SECTION

Assay of tetracyclic triterpenes in Chaga Tincture. Tincture (100 ml) was placed in a 250-ml round-bottomed flask and concentrated to about 50 ml. The concentrated extract was quantitatively transferred to a separating funnel, washing the flask with two 10-ml portions of water. The washing water was added to the main extract. Ethyl ether (70 ml) was added and the mixture was shaken for 1 min. After complete separation of layers, the ether extract was collected. Ether extraction was repeated a further three times using two portions of 70 ml and one of 30 ml. The combined ether extract was reduced in vacuo to a volume of about 60 – 70 ml, placed in a separating funnel, and treated with 5% caustic soda using portions of 10 and 5 ml. After separation of the phases, the alkaline solution containing phenol compounds was collected. Non-phenol substances, including tetracyclic triterpenes and steroids, remained in the ether extract. The ether fraction was washed with two 5-ml portions of water and was then filtered through filter paper bearing 5 g of anhydrous sodium sulfate. The filter was washed with 5 ml of ether. The ether extract was evaporated in vacuo to a dry residue, yielding a dry tetracyclic triterpene fraction. The dry residue was dissolved in 20 ml of 96% ethanol, transferred to a 100-ml measuring flask, and made up to the mark (solution A).

Solution A (5 ml) was placed in a 25-ml measuring flask and made to the mark with 96% ethanol (solution B).

Solution B (1 ml) was placed in a 15-ml tube with a ground-glass stopper, 1 ml of 10% vanillin solution in 96% ethanol was added and the solutions were mixed. Sulfuric acid (72%, 5 ml) was added. The reaction was held at 60°C for 10 min and then cooled to room temperature.

The optical density of the colored solution was measured on a spectrophotometer at a wavelength of 550 ± 5 nm in a cuvette with a 10-mm path length (Fig. 1).

Reference solution was prepared by placing 1 ml of 96% ethanol in a tube and adding 1 ml of 10% vanillin solution in 96% ethanol and 5 ml of 72% sulfuric acid, followed by processing as for the test solution.

The optical density of a standard lanosterol solution was measured in parallel. Reference substance lanosterol (Sigma-Aldrich L5768, 2008) (1 ml) was placed in a tube with a ground-glass stopper, 1 ml of 10% vanillin and 5 ml of 72% sulfuric acid were added, and subsequent processing was as for the test solution.

TABLE 1. Metrological Characteristics of the Method Used for Assay of Tetracyclic Triterpene Contents, Expressed as Lanosterol, in the Non-Phenol Fraction of Chaga Tincture.

n	$f(n-1)$	x	S^2	$\pm S$	P, %	$t_{(p,f)}$	$\pm \Delta X$	$\pm E$ %
10	9	0.0225	2.62×10^{-7}	5.12×10^{-4}	95	2.26	1.157×10^{-3}	5.14

TABLE 2. Lanosterol Addition Experiments.

Tetracyclic triterpenes found, expressed as lanosterol, g	Lanosterol added, g	Calculated, g	Found, g	Relative error, %
0.0225	0.0203	0.0428	0.0415	3.04
	0.0148	0.0373	0.0356	4.56
	0.0105	0.0330	0.0314	4.85

The content of tetracyclic triterpenes, expressed as lanosterol, was calculated as:

$$X = \frac{D \cdot m_{lan} \cdot 1}{D_{lan} \cdot 50 \cdot (1+1+5)} : \frac{100 \cdot 5 \cdot 1}{100 \cdot 25 \cdot (1+1+5)} \times 100 = \frac{D \cdot m_{lan} \cdot 10}{D_{lan}},$$

where D is the optical density of the test solution, D_{lan} is the optical density of the standard lanosterol solution, and m_{lan} is the weight of the standard lanosterol sample, g.

Notes

1. Preparation of standard lanosterol solution. About 0.008 g (accurately weighed) of standard lanosterol is dissolved with gentle heating in 20 ml of 96% ethanol in a 50-ml measuring flask and the solution is made up to the mark. The shelf life of this solution is one month.

2. Preparation of 10% vanillin solution. Vanillin (1 g, TU 6-09-10-544–76) is placed in a 10-ml measuring flask and dissolved in 5 ml of 96% ethanol with gentle heating and, after cooling, the resulting solution is made up to the mark with 96% ethanol.

3, Preparation of 72% sulfuric acid solution. Concentrated sulfuric acid (72 ml) is added to 28 ml of double-distilled water. The storage time is one month.

The metrological characteristics of the assay of really tetracyclic triterpenes, expressed as lanosterol, in Chaga Tincture are presented in Table 1.

Metrological analysis of the method showed that the relative error of a single estimate with a significance level of 95% was 5.14%.

The absence of systematic errors was demonstrated in experiments using addition of lanosterol (Table 2).

The tetracyclic triterpene content expressed as lanosterol in the non-phenol fraction of Chaga Tincture ranged from 0.01% to 0.035%.

Identity of Chaga Tincture.

Solution A (80 ml) prepared for assay was dried in vacuo the a volume of 1 – 2 ml (solution 1). Standard lanosterol solution (40 ml) was dried in vacuo to a volume of 1 – 2 ml (solution 2). Aliquots (0.02 ml) of solutions 1 and 2 were loaded onto Sorbfil PTSKh-P-V-UV plates (10 × 10 cm) as bands of about 2 cm. Loaded plates were placed in a chamber with a solvent mixture consisting of chloroform and methanol (25:1) and ascending chromatography was performed. When the solvent front reached 8 cm, the plates were removed from the chamber, dried in an air stream, and treated with Lieberman-Burchard reagent. Totals of 9 – 11 zones colored yellow and gray-violet appeared on the chromatogram. The yellow zone at the level of the standard lanosterol marker, with R_f about 0.8, corresponds to lanosterol in the tetracyclic triterpene fraction of Chaga Tincture (Fig. 2).

Note. Preparation of Lieberman-Burchard reagent. Concentrated sulfuric acid (5 ml) was added to 20 ml of cooled acetic anhydride slowly with mixing. The solution was stored at 5 – 6°C for three days.

Testing of Befungin showed that the content of the purified tetracyclic triterpene fraction, expressed as lanosterol, ranged from 0.01% to 0.02%. Experiments were performed using Befungin from OAO Tatkhimfarmpreparaty, ZAO Vifitekh, and OOO Kameliya NPP (experimental batches).

Tetracyclic triterpene contents, expressed as lanosterol, in propylene glycol extracts were about 0.02%.

Studies of crude chaga showed that the tetracyclic triterpene content, expressed as lanosterol, in the non-phenol fraction range from 0.35% to 0.8% per unit absolute dry weight.

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