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Standardization of *Citrullus colocynthis* (L.) Shrad. fruits dry extract for futher study of its antidiabetic activity

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KEYWORDS:
***Citrullus colocynthis*; dry extract; HPLC; standardization; validation; ellagic acid**

SUMMARY

Citrullus colocynthis (L.) Shrad. *(C. colocynthis)* is a perennial herb of Cucurbitaceae Family native in Arabia, West Asia, Tropical Africa and in the Mediterranean region. *C. colocynthis* is a perspective medicinal plant due to its pharmacological activities in particular for the treatment of diabetes mellitus. In this study a dry extract of *C. colocynthis* fruits was obtained. Technological scheme of extract obtaining was represened. Obtained dry extract has been standardized for these parameters: solubility, pH, weight loss on drying, the content of heavy metal, total ash content, identification and determination of quantitative content of biologically active substances (BAS). *C. colocynthis* fruits dry extract was standardized with the content of ellagic acid (EA) (0.62%). High performance liquid chromatography (HPLC) method was developed and validated for quantification of EA. The validated procedure is linear, specific, and therefore can be used to determine EA in *C. colocynthis* dry extract.

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1. Introduction

In recent years diabetes mellitus (DM) gains menacing proportions of the global pandemic. According to the International Diabetic Federation (IDF) by the year 2015 there were 415 million patients with diabetes, and according to experts in 2040 their number is expected to increase to 642 million people[1]. Today more attention is paid to phytotherapy of DM[2,3]. Herbal preparations, unlike synthetic ones, do not have a single mechanism of action. Their influence on the organism is caused by a balanced complex of biologically active substances (BAS), which simultaneously act on both the underlying disease and the functional disorders that accompany it. It should be noted that the total effect of the action of extracted substances may differ from the effect of the basic BAS, therefore, taking into account the expected pharmacological activity, the isolation of a specific physiologically active substance is not always necessary[4,5].

Colocynthis – representative of the Cucurbitaceae family, known for its antioxidant, antihyperglycemic, antihyperlipidemic, hepatoprotective properties, used for treatment of inflammatory processes, joint pain, fever, bacterial and fungal infections[6-10].

The largest number of preclinical and clinical studies is devoted to the study of *C. colocynthis* antidiabetic activity. In the traditional medicine of the Mediterranean countries *C. colocynthis* is known as

an antidiabetic agent[11,12]. Despite this literary sources contain rather contradictory information about the optimal form of plant administration, some of which supplement the results obtained earlier, others exclude them, which leads to the continuation and conduction of new trials[13-16].

In order to study antidiabetic activity of *C. colocynthis* we obtained a dry extract from its fruits, which was subsequently standardized, so reduced to a certain content of a substances with known therapeutic effect, to ensure that the object of standardization is in line with its functional purpose.

2. Materials and methods

The studied material was a dry extract obtained from *C. colocynthis* fruits in the laboratory of the Department of Pharmacognosy and Botany of Bogomolets National Medical University (Kyiv, Ukraine).

2.1. Obtaining of C. colocynthis fruits dry extract

Dry fruits of *C. colocynthis*, imported from Egypt (Cairo), were grounded to a particle size of about 0.5 mm through a sieve[17]. The crushed pieces of the fruits were extracted in a Soxhlet apparatus (extractant – chloroform)[18]. The extaction cake was dried, and then extracted with purified water for 30 minutes (in the ratio of 1:10) in a water bath[19]; the obtained extract was filtered; the filtrate was evaporated and dried to a residual moisture content of 5%. Thus, a dry extract of *C. colocynthis* fruits was obtained.

2.2. Standardization of C. colocynthis fruits dry extract

According to the Monograph «Extracts» of the State Pharmacopoeia of Ukraine (SPhU) for standardization the numerical parameters were determined in 5 batches of the extract obtained in laboratory conditions. The following indicators were determined: solubility, pH, weight loss on drying, the content of heavy metal, total ash content, identification and determination of quantitative content of BAS. The determination of solubility of dry extracts in various solvents, pH and the content of heavy metal were carried out according to the standard methods of the SPhU.

2.2.1 Weight loss on drying.

This indicator is introduced to control the content of volatile substances and / or moisture in the substance. According to the method *(2.2.32)* of the SPhU if there are no other indications in a separate monograph and the substance is not crystalline solvates, the weight loss on drying or the water content should not exceed 5%. Testing of the samples was determined after drying in a dryer «ШС-161». Approximately 0.2 g (precise weight) of the substance, with an accuracy of 0.002 g, was dried in a dryer at a temperature of (105 ± 1)° C to constant mass. The calculation was carried out according to the formula 1:

Weight loss on drying,

$$\% = (W1+W2) - W3 / W2 \times 100\ \%, \quad (1)$$

where W1 – the weight of the empty weighing bottle, mg;
W2 – weight of the tested sample, mg;
W3 – constant weight of the weighing bottle and sample after drying, mg.

2.2.2 Total ash.

The common ash was determined according to the method *(2.4.16)* of the SPhU after combustion of substances in a muffle furnace «МП-2У». Approximately 0.2 g (precise weight) of the substance, to an accuracy of 0.002 g, was placed in a crucible, dried to a constant mass. It was burned on a tile and placed in a high-temperature oven, burned at 600-650 ° C to constant weight (two weighings, a difference ≤ 0.0005 g). The calculation was carried out according to the formula 2:

Total ash, $\% = (W3-W2) / W1 \times 100\ \%$, (2)

where
W1 – weight of the tested sample, mg;
W2 – the weight of the empty crucible, mg;
W3 – constant weight of the crucible and ash, mg.

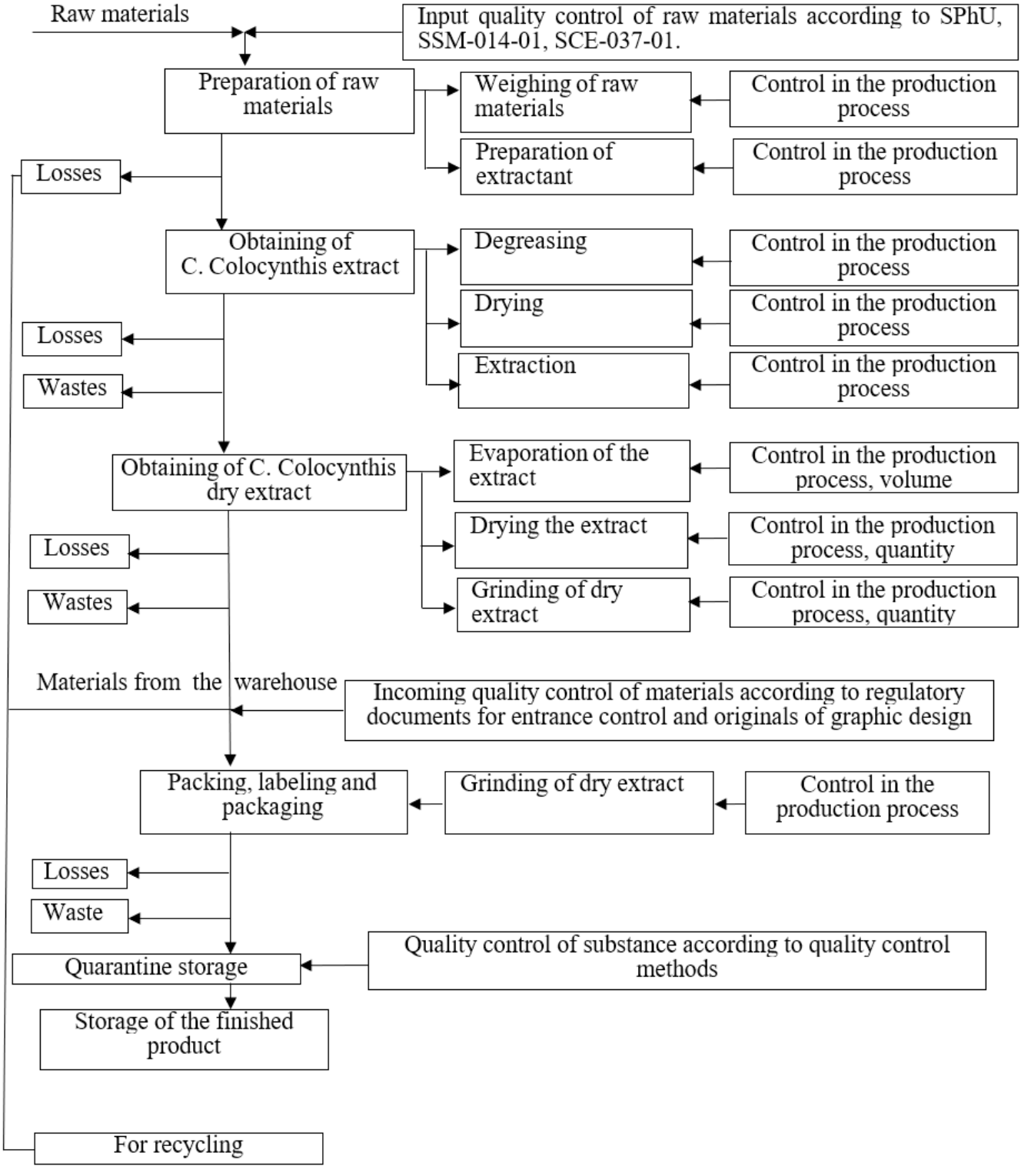

Figure 1. *Technological scheme of production of C. colocynthis dry extract*

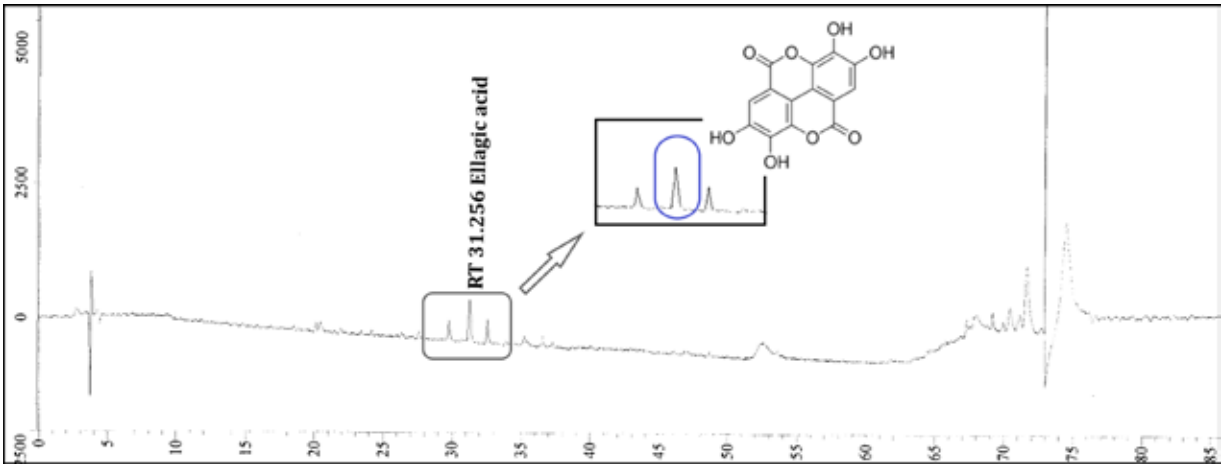

Figure 2. *Typical chromatogram of C. colocynthis dry extract solution*

2.2.3 Identification and qualitative determination.

For the identification of dry extracts methods of infrared spectroscopy or high-performance liquid chromatography combined with characteristic chemical reactions are commonly used. HPLC studies were performed on a Shimadzu LC20 Prominence liquid chromatograph in a modular system equipped with a four-channel pump LC20AD, column thermostat CTO20A, automatic sampler SIL20A, diode-matrix detector SPDM20A in comparison with the external standard model of elagic acid in such conditions:

- column Phenomenex Luna C18(2), size 250 mm x 4,6 mm, particle size 5 μm;
- column temperature – 35° C;
- detecting wavelength– 330 nm;
- flow rate of the mobile phase – 1 ml/min;
- volume of the entered sample – 5 μl;

Mobile phase: Eluent A: 0.1% solution of trifluoroacetic acid in water; Eluent B: 0.1% solution of trifluoroacetic acid in acetonitrile.

Identification of the components was carried out in accordance with the time of retention and compliance of the UV spectra with the substances-standards (at 254 nm and 330 nm). The HPLC method has been validated in accordance with accepted scientific practice and existing recommendations for analytical validation, conducted in accordance with the requirements of the ICH management regarding the validation of the Higher Technical Methods (HACCP): Text and Methodology Q2(R1)[20].

3. Results

The obtained extract is homogeneous composition, with a characteristic smell and a specific bitter taste, brownish-orange color. Technological scheme of extract obtaining is given in **Figure 1**.

In order to investigate the pharmacological activity of *C. colocynthis* dry extract, obtained for the first time, it was necessary to establish parameters for its standardization. Parameters of standardization of *C. colocynthis* dry extract are represented in **Table 1**.

According to results of standardization *C. colocynthis* dry extract is well soluble in water and practically insoluble in alcohol. All 5 samples corresponded the parameters of standardization. The average value of pH of 10% aqueous solution of *C. colocynthis* dry extract was 5.0, weight loss on drying was 2,71 %. The content of heavy metals was no more than 0.01%. The content of total ash in dry extract was set at 6.34%.

According to results of the HPLC the chlorogenic acid content (retention time = 20,5 min) was 0.05% and EA (retention time = 31,2 min) was 0.62% (**Fig.**

Table 1. Parameters of standardization of C. *colocynthis* fruits dry extract according to requirements of SPhU

		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Description	A homogeneous brownish-orange powder with a characteristic odor and a specific bitter taste	+	+	+	+	+
Solubility	Well soluble in water, practically insoluble in alcohol	+	+	+	+	+
Identification	When interacting with cupri-tartaric solution, a red brick-red precipitate develops (reducing sugars)	+	+	+	+	+
Weight loss on drying	< 4.0 %	3.02 %	2.83 %	2.10 %	2.46 %	3.15 %
pH	4.5-6.5	4.72	5.12	5.25	4.81	5.05
Total ash	< 9,0 %	5,32 %	6,15 %	7,04 %	5,20 %	8,01 %
The content of heavy metals	< 0.01%	<0.01 %	<0.01 %	<0.01 %	<0.01 %	<0.01 %
The content of ellagic acid	≥ 0.5 %	0.63 %	0.60 %	0.64 %	0.61 %	0.62 %

* + – Sample corresponds the parameter of standardization

2). Other phenolic components were unknown. Taking into account research data about positive impact of EA on the course of diabetes through different ways of action in particular suppressing the activity of phosphorylase and α-glucosidase, slowing of glucose transport through the intestine, insulin-tropic action, protection of SOD from glycosylation and fragmentation and others[21-28] the extract was standardized with the content of EA.

Validation parameters of the methodology for determining the EA by the HPLC method are given in **Table 2**.

4. Discussions

At the stage of preclinical experimental research medicinal plant raw materials are typically used in the form of various extracts. For the convenience to study pharmacological activity of *C. colocynthis* fruits we obtained a dry extract from a pre-obtained aqueous fruit extract. The dry extract has a number of advantages over liquid extract and soft extract. Due to low moisture content (no more than 5%), the dry extract is easily transported. Dry extract can be crushed to a powder state, exactly dosage, which contributes to increasing its therapeutic effect.

In search of the most effective form of application that determines the presence of a certain type of pharmacological activity based on the composition of BAS in the received substance, the researchers proposed to use different types of extracts from medicinal plant material of *C. colocynthis*13-16. Scientists have contradictory data on the benefits of different types of extracts, their safety and efficacy[29-31]. Marwat S. K. et al. in their review highlighted the positive effect of various seed extracts

Table 2. Generalized validation parameters of HPLC method

Parameter	Result
Maximum of absorption	330 nm
System suitability	Tailing factor = 1,19 RSD = 0,23 % Theoretical plates = 21047
Linearity	Range: 2-25 mg/ml Correlation coefficient r^2=0.9998; Linear equation: Yi = 0,9923x + 0,0057; Slope = 0.9923; Intersept = 0.0057
Specificity	Standard solution of EA: Rt EA = 31.2 min Placebo solution of EA: Rt EA = absent C. *colocynthis* dry extract solution of EA: Rt EA = 31,2 min The method is specific
Precision (%RSD)	2,23 % (NMT 3,0 %)
Intra-laboratory precision	2.89 % (NMT 3,2 %)
Accuracy	0.14 % (NMT 0,74 %)
Stability	0.01 (NMT 1,024 %)
Limit of quantitation (LOQ)	0.143% to the concentration of the standard solution
Limit of detection (LOD)	0.047 % to the concentration of the standard solution

of *C. colocynthis* (aqueous extract, fat-free aqueous extract, aqueous methanolic extract, ethyl acetate and n-butanol extracts) on the following indicators: glucose tolerance, body weight, mass of the pancreas, diaphragmatic muscle tissue, serum cholesterol, triglycerides, urea, creatinine, transaminase and alkaline phosphatase in animals with diabetes. The authors concluded that the most pronounced effect in diabetic rats had aqueous and n-butanol extracts, the lowest – fat-free aqueous extract[32].

Investigation of phenolic compounds content was a priority for us, since it is known that they possess antioxidant, antiinflammatory and also antidiabetic properties[8,31,33,34,35,36].

Rashedi H. et al. shows a significant difference in the content of flavonoids and phenolic compounds for different parts of *C. colocynthis*. The maximum content of flavonoids and phenolic compounds were observed in fruits and stems. Moreover, the content of these compounds is different depending not only on a certain anatomical part of the plant but also on the type of extract. Such facts have been proven in studies by E. Chekroun et al., A. I. Hussain et al., N. Benariba et al.

The object of our research was *C. colocynthis* fruits dry extract. We detected the presence of EA and HA in the fruits of *C. colocynthis* by HPLC method. Hussain A. I. et al. by the method of reverse phase high performance liquid chromatography (RP-HPLC) found ferulic acid, vanillic acid, p-coumeric acid, gallic acid, p-hydroxy benzoic acid and chlorogenic acid, and flavonoids quercetin, myricetin and catechin in ethanol and hexane extracts of roots, leaves and fruits. The data of the author according to the

results of the chromatographic analysis coincide with ours regarding the presence of chlorogenic acid in fruits of *C. colocynthis*. However, in the list of compounds mentioned by the author there is no elagic acid, which was obtained in our trials. Both the author and we carried out the determination of phytochemical compounds by HPLC method which was developed and validated31. In trials of other scientists' identification of phenolic compounds was carried out using the method in which Folin-ciocaltue was used[34,36].

Obtained results regarding the component composition of *C. colocynthis* fruits dry extract were expected. Our previous research on the culture of pancreatic cells of Rin-m5F showed the presence of pronounced antioxidant properties of extract, which contributed to the increase in the number of living cells in the culture in the environment of prooxidant factors action[37]. We believe that this effect was caused by EA, which is a strong antioxidant of natural origin. The ability of *C. colocynthis* fruits extract to reduce glucose levels in in vitro and in vivo experiments[37,38] is also likely to be in most cases related to the effects of EA.

A number of studies confirm the antidiabetic activity of EA through various mechanisms of its action[21-28]. In the literature review, Gurudeeban S. et al. the antidiabetic effect of the *C. colocynthis* is also associated with the content of phenolic compounds, namely, flavonoids (isorientine and isovitexin)[39]. Some scientists argue that flavonoids have the ability to stimulate the synthesis of insulin in vitro, including apigenin[40] and quercetin[41].

In any case, it is necessary to provide further researches to investigate the pharmacological activity of *C. colocynthis* dry extract to understand the mechanism of its antidiabetic activity. Another important question is the condition of raw materials of medicinal plants[42]. So, it is necessary to carry out further research of the condition of raw material resources of wild growing *C. colocynthis* in Ukraine for its probable use in medicine and pharmaceutical industry.

5. Conclusions:

1. For the first time a technology for obtaining a dry extract of *C. colocynthis* fruits has been developed. *C. colocynthis* fruits dry extract was standardized with the content of ellagic acid (0.62%).

2. Methodology for determining the ellagic acid in *C. colocynthis* fruits dry extract by the HPLC method was validated. The validated procedure is linear, specific, and therefore can be used to determine elagic acid in a dry extract of *C. colocynthis.* □

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