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Article

Application of Quality by Design Approach to the Pharmaceutical Development of Anticancer Crude Extracts of *Crocus sativus* Perianth

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Abstract: The application of the Quality by Design (QbD) concept to extracts obtained from *Crocus sativus* perianth with potential anticancer activity will ensure the safety, efficiency, and quality control of the entire technological process, as well as determine the critical factors affecting the quality of extracts. Potentially critical points of the production of the plant extracts, including the cultivation and processing of the plant materials, the extraction process, and the choice of solvents, were identified using the Ishikawa diagram and FMEA risk assessment methods as well as the corrective actions proposed. The Herbal Chemical Marker Ranking System (HerbMars) approach was used to justify the Q-markers choice of *Crocus*, which takes into account bioavailability, pharmacological activity, and the presence of the selected standard. An experimental design (DoE) was used to assess the influence of potentially critical factors on the efficiency of the compound extraction from raw materials with water or ethanol. The presence of 16 compounds in *Crocus* perianth was determined by HPLC and their quantitative assessment was established. Selected compounds (ferulic acid, mangiferin, crocin, rutin, isoquercitrin) can be used for the quality control of *Crocus* perianth. In addition, the stigmas from the Volyn region met the requirements of ISO 3632 for saffron as a spice (category I). The cytotoxic activity against melanoma (IGR39) and triple-negative breast cancer (MDA-MB-231) cell lines of the hydroethanolic extract of *C. sativus* perianth was significantly more pronounced than the water extract, probably due to the chemical composition of the constituent components. The results show that the QbD approach is a powerful tool for process development for the production of quality herbal drugs.

Keywords: Quality by Design; risk assessment; design of experiments; Herbal Chemical Marker Ranking System; failure mode and effect analysis; saffron; anticancer herbal drugs

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1. Introduction

Despite the increasing popularity of herbal drugs, there are a large number of reviews [1–3] describing the adverse effects on the patient's health due to changes in the quality, effectiveness, and content of biologically active compounds in them. Herbal drugs, like any drug, must meet quality, safety, and efficacy requirements. The implementation of the ICH Q8, Q9, and Q10 guidelines has combined the stages of pharmaceutical product development, where quality assurance of the medicinal product is laid down at each stage of production by the requirements of Good Manufacturing Practice (GMP). In this regard, the World Health Organization (WHO) has developed several guidelines for the

standardization steps of herbal products [4]. The Quality by Design (QbD) concept is a part of the ICH Q8 (R2) "Pharmaceutical Development" guideline and ensures product quality by modeling and controlling the manufacturing process itself, and not just relying on the final product at the end of the process to be the quality test [5,6]. This approach allows the development of reliable, controlled processes and operations, which results in a quality product. The methodological approach based on the QbD was applied in the current work to plan the process of obtaining plant extracts from *Crocus sativus* perianth with anticancer effect.

Saffron is one of the most valuable spices in the world, which is cultivated in several countries for the food industry. Saffron, in filaments, is the dried dark-red stigmas of the *C. sativus* (Iridaceae family plant) flower. The stigmas are separated from the perianth, and all other parts of the flower are a by-product [7]. It takes approximately 150,000 flowers to produce 1 kg of *Crocus* stigma. However, *Crocus* perianth contains various biologically active compounds and can be considered as a potential medicinal raw material. A review of scientific articles showed that *C. sativus* flower extracts have different pharmacological activities: antibacterial (125 mg/mL) [8,9], antioxidant, and free radical scavenging properties [10,11]; is antiproliferative (ED_{50} 0.42 mg/mL) against Caco-2 cells [12]; antityrosinase activity (50 µg/mL); individual compounds, such as crocin 1 and safranal have inhibitory activity against human monoamine oxidases [13]; etc. Saffron, as a spice, is also grown in the Ukraine [14], where there is a large amount of raw perianth after *Crocus* stigma processing.

Since *Crocus* raw material from the Ukraine is a new source for the production of herbal drugs, it is necessary to evaluate its chemical composition. The chemical composition of herbal raw materials is usually variable and depends on environmental and technological factors, so it is important to ensure control during the production of the product. Harvesting, drying, storage, transportation, and processing methods (e.g., the extraction method and the polarity of the extracting solvent, component instability, etc.) have a particular impact on the quality of the starting herbal raw material [15]. However, for *C. sativus* raw material standardization, parameters have not been developed. Assessment of the quality control of herbal raw materials, extracts, and drugs based on active markers is mandatory. Quality markers are "chemically defined constituents or groups of constituents of an herbal substance, an herbal preparation or herbal medicinal product that serve for quality control purposes, independent of whether they have any therapeutic activity". The EMA describes two different categories of chemical/quality markers. The constituents of an herbal medicine responsible for its therapeutic activity or active markers and the constituents that are characteristic for its taxon or analytical markers [16].

The Herbal Chemical Marker Ranking System [17] was proposed for the selection of quality markers (Q-markers) for herbal raw materials and preparations. This approach takes into account the bioavailability, the reported bioactivity, the quantitative content of the metabolite, and its physiological effects associated with the intended use of the raw material, as well as the commercial availability of the standard. It is important for the Q-marker to be able to trace it throughout the entire production process, from raw materials, then obtained extracts, to finished products.

Thus, the current work aimed was to apply the QbD concept to experimental design; analysis of current processes and risk management to ensure the quality of obtained plant extracts; determination of the most appropriate chemical markers for quality control of *C. sativus* perianth and extracts; development of a quality assessment method for multicomponent analysis; fingerprinting of *C. sativus*; assessment of the antioxidant and cytotoxic potential of the obtained extracts.

2. Results

2.1. Used of QbD Approach

The "Quality by Design" (QbD) concept originated in the field of quality management and has recently been applied to the process planning and manufacturing of pharmaceuti-

cals [18]. It is defined in ICH Q8 Pharmaceutical Development as a systematic approach to development that starts with predetermined aims and focuses on product and process understanding and process management based on sound science and quality risk management. The European Medicines Agency (EMA) [6,16] adapted the QbD approach to improving understanding of the herbal drugs manufacturing process. However, despite the advantages of this modeling, the QbD approach has not yet been fully implemented at the planning and manufacturing stage of drugs. The philosophy and approach of QbD were applied to optimize the targeted search for anticancer compounds on the example of obtaining extracts from *Crocus* perianth.

The general workflow for the development of an herbal extract based on the application of the QbD approach, adapted for *Crocus* perianth, is shown in Figure 1. This approach guarantees a reliable process from the selection of herbal raw materials, extraction for herbal drug production, even if the starting material of herbal origin has a wide variety, which is typical for plant materials.

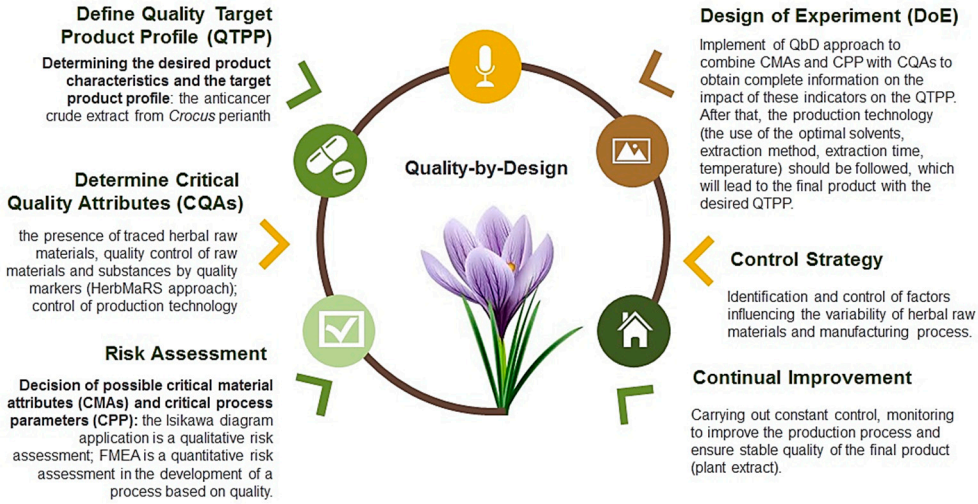

Figure 1. The QbD approach to quality assurance in *Crocus* perianth drugs production. Adopted by [5,6,19].

According to the QbD approach, pharmaceutical development should include at least the following elements and phases:

- determination of the desired characteristics or the Quality Target Product Profile (QTPP);
- identification of potentially Critical Quality Attributes of a drug (CQAs);
- determination of possible Critical Process Parameters (CPPs) and the pharmaceutical substance characteristics and excipients (Critical Material Attributes (CMAs));
- development and implementation of Design of Experiment (DoE), its optimization, the definition of control strategies, and improvements.

To develop the desired herbal drug substance by the ICH Q8 guidelines, the "Quality Target Product Profile" was compiled. It is based on quality indicators for anticancer herbal preparations, namely, high efficiency, low toxicity, and the possibility of long-term use in therapy [4,20–22]. Herbal compounds have a positive effect on the survival, immunomodulation, and quality of life of cancer patients, in combination with traditional therapeutic agents. The main compounds of *Crocus* flowers are phenolic compounds [7,10,11], which exhibit pronounced antioxidant properties. Therefore, the ultimate aim of the process is to obtain a high-quality plant extract from *Crocus* perianth with pronounced anticancer activity. To ensure the properties specified in the QTPP, a method was chosen to obtain extracts with different solvents to determine the most suitable extractant based on the results of chromatographic analysis.

Further, the Critical Quality Attributes (CQA) of the selected product [19] should be determined, which provide its desired quality with proper control during the production process. CQAs include physical, chemical, biological, or microbiological properties or characteristics of the starting plant material as well as the obtained extract. Following the QbD approach, CQAs are dynamic characteristics of the process development that need to be updated and improved during the product life cycle [5]. A risk analysis is used to identify points of process control to determine the CQAs. These CQAs are designed to obtain standard plant raw materials by complying with the GACP recommendations for plant cultivation, which will provide the traceability of herbal raw materials and guarantee a stable result of pharmacological activity. In addition, the critical points of the process are the quality control of raw materials, auxiliary materials, and the extraction process to ensure the plant extract is of consistent quality and composition.

2.2. Risk Assessment of Crocus Perianth Extracts Production

Risk assessment is part of risk management and should be conducted early in process development. Once the QTPP has been defined and the CQAs have been identified, the risk assessment and design space identification can be obtained either through experimentation or modeling. The guideline ICH Q9 "Quality Risk Management" provides a list of nine common risk management tools, namely, basic risk management facilitation methods (Ishikawa fishbone diagram, flowcharts, check sheets, etc.); fault tree analysis; risk ranking and filtering; preliminary hazard analysis; hazard analysis and critical control points; failure mode and effects analysis (FMEA); failure mode, effects, and criticality analysis (FMECA); hazard operability analysis; supporting statistical tools. According to the QbD conception, risk assessment has priority over DoE. Among the most commonly used tools are Ishikawa's fishbone diagram and FMEA [23].

An Ishikawa diagram identifies and groups different kinds of effects, such as material properties, equipment design, and process parameters, that may pose a risk to certain CQAs, such as yield, purity, or overall processability [4,21,22]. The main branches of the fishbone are subdivided into sub-branches that reveal a more detailed causal relationship between potential cause and risk. This diagram may already identify critical process parameters (CPPs) that must be maintained within a certain range during the process and, therefore, must be part of a process control strategy and may require further study. An approximate early risk assessment for the procedure for obtaining plant extracts with a stable composition of components from *Crocus* perianth is shown in Figure 2.

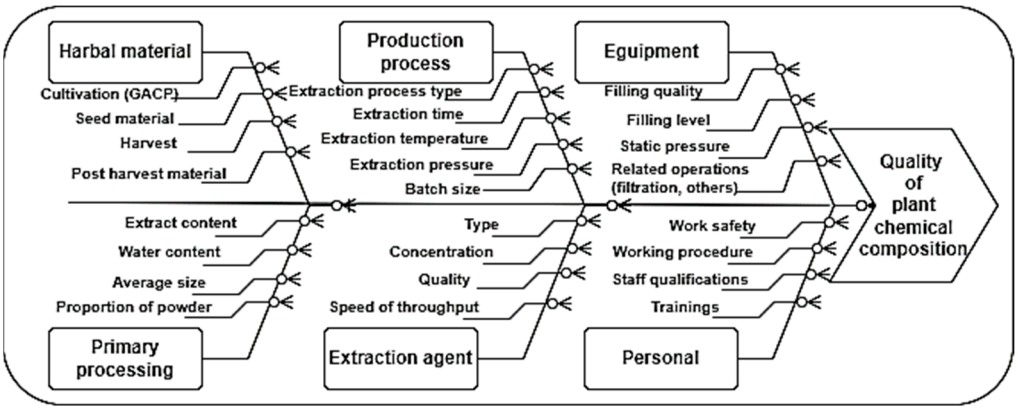

Figure 2. Risk assessment of *C. sativus* perianth extracts obtaining using the Ishikawa method.

The main risks of plant materials obtained with a stable chemical composition are the biological and physiological aspects of plant cultivation [24]. The environment during the cultivation and harvesting can lead to very different compositions of active compounds. In this regard, WHO proposed to adhere to the GACP recommendations on the possibility of traceability and obtaining the quality of starting raw material of plant origin [25]. In a

previous study, it was shown that the *C. sativus* plant in the Kherson region, Ukraine, is grown in compliance with the developed and implemented Standard Operating Procedures for this crop, taking into account the climatic characteristics of the country [14]. Primary processing also has norms and requirements. A similar approach to cultivating saffron was used in the farm of the Volyn region, which guarantees the receipt of high-quality raw materials at the first stage of development. As a result of processing *Crocus* flowers and extracting its stigmas, a large mass of perianth is formed, which is production waste [12].

It can be seen that six main reasons have been identified in the current experiment, including the starting raw material of plant origin, primary processing of raw materials, extraction conditions, equipment, and the availability of trained personnel and associated sub-reasons. The first step is cultivating the plant in compliance with the GACP principles and primary processing of raw materials, then preparing the raw materials for extraction and conducting analysis to establish its chemical composition by HPLC. The impact of human error, equipment, and the environment on quality can be reduced through effective management and by adhering to standard operating procedures or maintenance schedules. The risks associated with the process and materials, in this case, obtaining quality feedstock and the process of selecting an extractant, are the most important for the characterization of the process [26].

The FMEA was used to quantify specific risks. FMEA, for the development of the *Crocus* perianth plant extract production process, is derived by evaluating the range of CPP defined by the Ishikawa diagram for the potential impact of risks that could affect quality indicators and the possibility of risks during the extraction of plant materials with solvents [27,28]. The preparation of the extract consists of several stages of primary processing, drying, grinding, extraction, and the actual obtainment of dry extracts from *Crocus* (Table 1). Analysis of failure mode and effect in *Crocus* extract production helps to prioritize risks and determine corrective actions to avoid identified problems.

Table 1. Failure mode and effect analysis of *Crocus sativus* perianth extract manufacturing process.

Risk Area, CCP/QCP	Failure Mode	Potential Cause or Route of Failure	Detection or Control Method	Risk Analysis				Correction Action
				S	P	D	RPN	
Herbal raw material QPT	• Cultivation • Seed material • Harvest • Post-harvest material	Poor cultivation management, inappropriate soil and irrigation; physical properties; long duration of handling and transporting, warm and humid condition; poor personal hygiene in collection; wrong handling by personnel.	Compliance with GACP principles of all cultivation processes; certified suppliers with HACCP program; documenting; visual inspection; botanical identification; soil analysis; metal detector; microbiological analysis.	5	4	4	80	Rejection, sorting, instructions to supplier
				4	3	3	36	
				5	4	4	80	
				5	4	5	60	
Primary processing CCP	• Extract content • Water and ash content • Average size • Proportion of powdered	Improper control of temperature and time of drying. operator error, poor development; material variation.	Calibration of thermometer and timer, maintenance program; personnel training; visual control; monitoring; microbiological analysis; chemical analysis.	5	4	5	100	Re-dry, sorting out; repair and replace damaged equipment
				5	3	5	75	
				4	3	2	24	
				4	3	2	24	
Extraction agent CCP	• Type • Concentration • Quality • Speed of throughput	Uniformity of the extractant concentration; content of extractive compounds; ethanol concentration; operator's error.	Alcoholometry; monitoring; chemical analysis; calibration; temperature control.	5	4	4	80	Instruction to operator
				5	4	5	100	
				5	4	5	100	
				5	3	3	45	

Table 1. *Cont.*

Risk Area, CCP/QCP	Failure Mode	Potential Cause or Route of Failure	Detection or Control Method	Risk Analysis				Correction Action
				S	P	D	RPN	
Production process CCP	• Extraction process type • Extraction time • Extraction temperature • Extraction pressure • Batch size	Poor monitoring; operator's error, equipment failure; machine failure, poor development; improper control of temperature and time, improper sealing of system; uniformity of the raw material.	Calibration of thermometer and timer, maintenance program, personnel training; monitoring; visual control; temperature control.	5	4	3	60	Repair and replace damaged equipment
				5	4	3	60	
				5	4	4	80	
				5	4	3	60	
				5	4	3	60	
Equipment QPT	• Filling quality • Filling level • Static pressure • Related operation (filtration, drying)	Defective devices for extraction, evaporation and drying.	All equipment should be easily cleaned to minimize contamination; calibration of equipment, personnel training; monitoring; visual control; temperature control.	4	2	1	8	Repair and replace damaged equipment
				3	2	1	6	
				3	2	1	6	
				3	2	1	6	
Personal QPT	• Work safety • Working procedure • Staff qualification • Trainings	Human error; poor personnel hygiene, wrong handling by personnel.	All persons having contact with raw materials should observe a strict level of personal hygiene; personnel training; monitoring; visual control.	4	2	1	8	Training, instruction to operator
				4	3	2	24	
				5	3	2	30	
				4	3	2	24	

Adapted according to [29]: S—severity of excursion = 1 (low), 5 (high); P—probability of occurrence = 1 (low), 5 (high); D—detection of probability = 1 (easy), 5 (hard); risk priority number RPN = S × O × D. 1–29 low risk, 30–59 medium risk, 60–125 high risk; CCP, critical control point; QCP, quality control point. The rank for risk quantification of the S, P, and D parameter is presented in Supplementary Table S3.

The results of calculating the final risk priority number (RPN) made it possible to classify possible inconsistencies in the technological process at the extraction stage as unacceptable risks. The risks arising at the stage of raw material preparation, purification, and extraction of extracts have a significant impact. In the process of risk management, the risk level was assessed and methods of risk control and prevention were proposed.

The Hazard Analysis and Critical Control Points System (HACCP) helps to identify key processing steps of plant materials to minimize the risk of microbial contamination and should be performed by default. HACCP implementation risk analysis, integrated with FMEA, helped in the current study to predict the presence of probable hazards, including biological (microorganism, enzymatic activity), chemical (pesticides residues, mycotoxins), and physical (heavy metals, foreign matter, critical moisture, browning, excessive water activity, dust) that can occur at different stages of the process. In the production of *Crocus* perianth extracts, the primary processing stage of herbal raw materials, including harvesting, sorting, and drying, no matter what type of drying process is used, is considered CCP. In addition, the extraction process is also a defining stage of production. Thus, the production of herbal drugs should be carried out in compliance with the GMP, GACP, and HACCP guidelines with strict control of all types of risks.

2.3. Design of Experiment

The aim of the DoE was to gain a deeper understanding of the processes and planning of the current experiment (Figure 3). The plan of the experiment included: defining the problem (the current problem of cancer diseases in the world), choosing the direction of solving the problem (searching for herbal preparations with anticancer activity), then justifying the choice of the object of research (*Crocus* perianth–selection of herbal materials with a wide raw material base and a promising composition of compounds according to the literature search). Further, the definition and selection of quality markers for the possible quality control of the starting plant material, obtained plant extracts, and, subsequently, the herbal drugs were carried out. Therefore, we applied the Herb MaRS approach to

select potential quality markers specifically for *Crocus* perianth extracts with anticancer properties. After that, the DoE provides for the chemical analysis of raw materials and obtaining extracts from the selected type of raw materials and their chemical analysis. As the final stage of the experiment, pharmacological tests of the obtained extracts for the presence of antioxidant and cytotoxic activity were carried out.

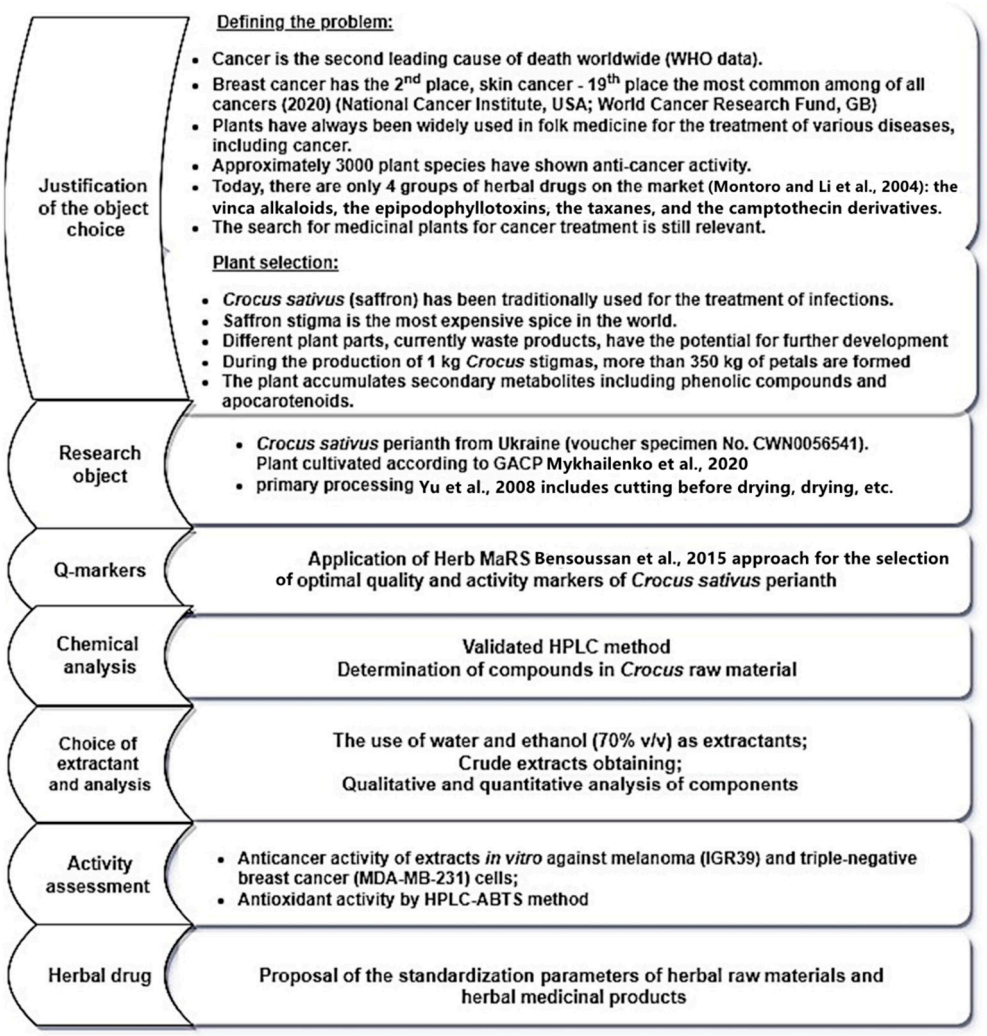

Figure 3. DoE of obtaining *C. sativus* crude extracts with potential anticancer activity [14,17,25,30,31].

The study also included the determination of the quality of *Crocus* stigmas in accordance with ISO 3632. The quality of saffron depends on the concentration of the main metabolites, crocin, picrocrocin, and saffron, which are responsible for the color, flavor, and aroma of the spice. The availability of high-quality raw materials of *Crocus* stigma motivates farmers to expand the areas of cultivation and production of the spice itself. As a by-product of the technological process, a large biomass of perianth is formed, which is the main object of study in the current work. In this regard, farming in the Volyn region is very promising. The area of the plantation is almost 4 hectares. Results of crocins ($E^{1\%}_{1cm}$ 440 nm was 253), safranal ($E^{1\%}_{1cm}$ 330 nm was 30), and picrocrocin ($E^{1\%}_{1cm}$ 257 was 95) amounts and moisture content (9.5%) of saffron showed that the tested sample fulfilled the ISO specifications for category I regarding moisture and the main spectrophotometric characteristics.

2.4. Selection of Crocus Perianth Q-Markers with the Herb MaRS-Approach

The choice of chemical markers is critical for the quality control of herbal raw material and drugs, as well as for identification and authentication. The most correct are therapeutic components (active compounds), that is, those components of the plant that determine its pharmacological activity. For plant materials, as a rule, there are several quality markers since the substances act synergistically. The choice of the chemical markers for quality assurance of the herbal raw material and crude extracts of *Crocus* perianth was made by using the Herbal Chemical Marker Ranking System (Herb MaRS) based on anticancer bioactivity against melanoma (IGR39) and triple-negative breast cancer (MDA-MB-231) cell lines. The innovative Herb MaRS procedure, for the identification of relevant chemical markers in complex medicinal plants, was developed by the National Institute of Complementary Medicine [17] and takes into account the bioavailability of the compound, the declared bioactivity and physiological effect associated with its intended use (in this experiment—anticancer), as well as the availability of a reference compound for analysis (Figure 4). In this work, the Herb MaRS approach was applied not to the collection from different herbs, but to one type of raw material (*Crocus* perianth) and to its various compounds. This approach was used to select quality markers based precisely on the approach of the system. Based on the Herb MaRS criteria, 19 compounds were selected, which were found in the raw material of *C. sativus* according to the various literature data [32–34].

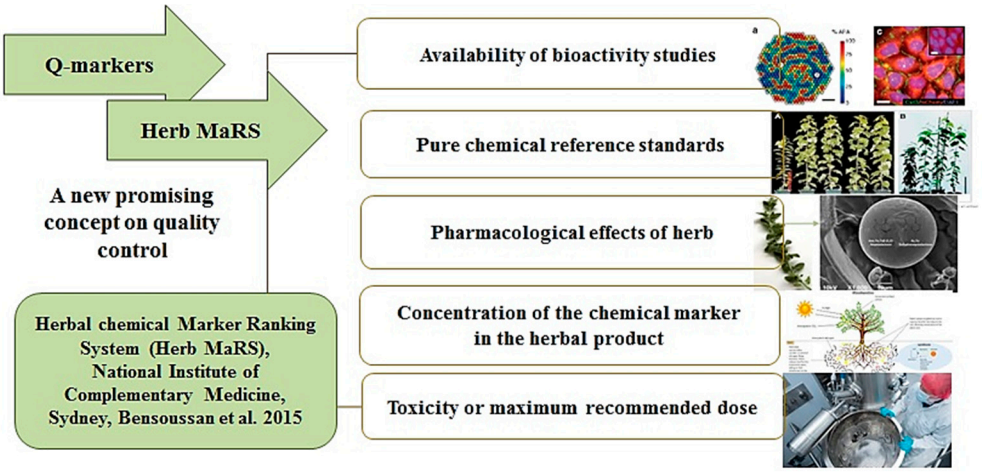

Figure 4. Herb MaRS criteria for chosen of Q-markers for *Crocus* perianth raw material and crude extracts.

The main components of *Crocus* stigma are crocins, safranal, and picrocrocin. They were also found by various authors in *Crocus* flowers [10,35]. In addition, cinnamic acid derivatives [35] and different flavonoids and their derivatives [10,12,34,36,37] have been found in various parts of *Crocus* (flowers, leaves, stigma). The qualitative and quantitative composition of phenolic compounds differ depending on the cultivation place and harvesting of *Crocus* raw materials. In addition, Table 2 shows isoflavonoids (nigricin, iristectorigenin B, tectorigenin), which are characteristic of plants of the *Iridaceae* family [38] and have previously been found in the leaves and stigmas of *Crocus* stigmas [39,40]. The compounds presented in Table 2 can be considered as potential Q-markers of *C. sativus* perianth in terms of relative importance in the treatment of cancer, provided they are found in the studied samples. In addition, all discussed compounds are available standards.

Table 2. Composition of *Crocus sativus* perianth and relevant Herb MaRS score based on potential anticancer action.

Compound	Activity	Herb MaRS Ranking *	Reference
All crocins	Anticancer, cytotoxic, antioxidant, neuroprotective, retinal damage protection, antidepressant, anti-Alzheimer, hypolipidemic, anti-inflammatory.	5	[41–51]
Picrocrocin	Anticancer, antineoplastic, antioxidant.	4	[45,52]
Safranal	Antitussive, anticonvulsant, antioxidant, antianxiety, antidepressant, antinociceptive, anti-ischemia.	3	[53,54]
Ferulic acid	Anticancer, anti-inflammatory, antioxidant, antibacterial, antidiabetic.	5	[55–58]
Caffeic acid	Anticancer, antioxidant, anti-inflammatory.	5	[59,60]
Mangiferin	Anticancer, antiviral, anti-inflammatory, antidiabetic, antitumor, lipometabolism regulating, cardioprotective, antihyperuricemic, neuroprotective, antioxidant, antipyretic, analgesic, antibacterial, immunomodulatory.	5	[61–67]
Isoorientin	Anticancer, anti-inflammatory, QS inhibitor, antinociceptive, gastroprotective.	5	[68–70]
Kaempferol-3-*O*-sophoroside	Antiinflammatory, antitumor, antioxidative, antiallergic, antidiabetic.	4	[71]
Rutin	Anticancer, anti-inflammatory, QS inhibitor, antibacterial, antiprotozoal, antitumor, antiallergic, antiviral, cytoprotective, vasoactive, hypolipidaemic, antiplatelet, antispasmodic, antihypertensive.	5	[72–77]
Isoquercitrin	Anticancer, antioxidant, antiproliferative, anti-inflammatory, anti-hypertensive, antidiabetic.	5	[78–80]
Apigenin	Anticancer, antiallergic, anti-inflammatory, antioxidant, antimutagenic, anticarcinogenic.	5	[81–84]
Apigenin-7-*O*-glucoside	Cytotoxic effect, antifungal, anticancer, antiproliferative.	5	[85–87]
Quercitin and its derivatives	Anticancer, antiviral, antiprotozoal, antimicrobial, antiallergic, anti-inflammatory.	5	[88–91]
Kaempferol and its derivatives	Antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic.	5	[92–96]
Astragalin	Anticancer, anti-inflammatory, antioxidant, neuroprotective.	4	[97,98]
Isoramnetin	Anticancer, cardiovascular, cerebrovascular protection, anti-inflammatory, antioxidant.	4	[99,100]
Nigricin	High anti-inflammatory activity.	3	[101]
Iristectorigenin B	Liver X receptor modulator, anti-inflammatory, antioxidant.	4	[102,103]
Tectoridin	Anticancer, anti-inflammatory, antioxidant, hepatoprotectivy, hypoglycemic, antiallergic, anaphylaxis inhibitory.	5	[104–106]

* The ranking score ranges from 0 to 5, with 0 being the least and 5 being the most suitable.

2.4.1. Traditional Saffron Used

Saffron is used in folk medicine in different countries for the treatment of cancer, eye diseases, disorders of the uneven system, and for normalizing metabolism [107]. Saffron has been used as a remedy for various diseases, including cancer, in ancient Arabic, In-

dian, and Chinese cultures. Saffron has traditionally been used for convulsions, asthma and bronchospasm, menstrual irregularities, liver disease, and pain. However, the main application was as a stimulant, aphrodisiac, and antidepressant [33]. As a homeopathic remedy, saffron is also presented in various pharmacopoeias of the world [108]. During the production of saffron, large masses of perianths are formed, which are a by-product of the production of spice. As a result of the analysis of the chemical composition of the perianth, as well as extracts from it, the presence of various compounds was established. The relevant compounds identified in the *Crocus* raw material are shown in Table 2 along with their activity and the corresponding Herb MaRS score.

2.4.2. Current Pharmacology Application of Compounds

The anticancer activity of flavonoids in vitro, as well as their chemopreventive potential in vivo, have been long known. For example, antitumor activity against MDA-MB-231 cells [70], as well as melanoma, leukemia, and erythroleukemia for rutin, kaempferol, and quercetin has been previously documented [74,93,94]. Rutin (20 μM) significantly ($p < 0.05$) increased the cytotoxic activity in MDA-MB-231 cells of the chemotherapeutic agent's cyclophosphamide and methotrexate. In another study, rutin at a dose of 30 mg/kg significantly reduced the growth of TNBC MDA-MB-231/GFP cells [75]. Administration of rutin at a dose of 10 mg inhibited the formation of B16 melanotic melanoma in C57BL/6 mice by more than 40% [71].

Crocins are the main chemical constituents of *Crocus* stigma. However, crocins are also found in *Crocus* flowers. Several studies have shown that crocins exhibit antitumor effects on various cancer cell lines [45,46]. Crocetin, *trans*-crocin-4, and safranal were reported to significantly inhibit the proliferation effect against breast cancer MDA-MB-231 (200 μg/mL) [47]. Crocin-induced apoptosis and cell cycle arrest in the G2/M phase in MDA-MB-231 cells in a dose-dependent manner (IC_{50} 5.97 mg/mL) [48]. For the *Crocus* stigma water extract, an antitumor effect was revealed in vivo on a highly metastatic murine B16-F10 melanoma cell line [49]. There are studies [50] on breast cancer cells MCF-7 and MDA-MB-231 showing the concentration-dependent inhibition of proliferation by crocetin, the main metabolite of crocins. Crocin is suggested to be one of the most effective components of saffron for cancer treatment.

Apigenin at concentrations of 25 μM, 50 μM, 75 μM, and 100 μM inhibited the viability of MDA-MB-231 cell by 12%, 27%, 42%, and 49%, respectively [81]. A potent antiproliferative effect of apigenin was shown against the human melanoma A375 cell line (EC_{50} 33.02 μM) [82]. In our previous experiment, apigenin showed the highest activity against melanoma IGR39 and breast cancer MDA-MB-231 cell lines (EC_{50} values were 131.8 ± 7.2 μM and 123.4 ± 19.0 μM, respectively) [39]. In other studies, apigenin-7-*O*-glucoside showed a more cytotoxic effect on colon cancer HCT116 cells compared to apigenin [83]. These data demonstrate that the sugar moiety of apigenin-7-*O*-glucoside has an important effect on the biological activity of apigenin.

Ferulic acid showed antitumor activity in various types of cancer such as colon and lung cancers, as well as tumors of the central nervous system. The authors showed a pronounced cytotoxic activity of ferulic acid in the MDA-MB-231 breast cancer cell line. The use of ferulic acid (3, 10, 30, and 100 μM) led to a decrease in viability, an increase in apoptosis, and suppression of the metastatic potential [54]. In another assay, ferulic acid (at a concentration of 120 μM) showed a pronounced cytotoxic activity against human skin melanoma cells (SK-MEL-3), significantly reducing cell viability compared to the control [56].

The pronounced antitumor potential of mangiferin was established in models of the breast cancer MDA-MB-231 cell line in vitro and in vivo [62]. Thus, mangiferin has immunomodulatory, antioxidant, anti-inflammatory, antiviral, antidiabetic, and anticancer properties [59,61]. Additionally, the anticancer effect of mangiferin has been confirmed in mouse models at doses between 5 and 10 mg/kg against ascitic fibrosarcoma [63],

50 and 100 mg/kg (oral) against benzo(a)pyrene-induced lung carcinogenesis [64,65], and 100 mg/kg (oral) against ER-negative breast cancer.

These and other compounds determine the anticancer potential of *C. sativus* [12,31]. Therefore, each of these compounds can be considered as a potential Q-marker of activity. The next step of the experiment was to determine the presence of the selected compounds in raw materials and extracts of *Crocus* perianth from the Ukraine. It is necessary to take into account the quantitative content of each component, its therapeutic activity, and the availability of a reference standard so that it can be proposed as a quality marker according to the chosen approach.

2.5. Assessment of C. sativus Perianth and Its Crude Extract Chemical Composition

2.5.1. Perianth Raw Material

A combined method using HPLC fingerprints and quantitative analysis to assess the stability of the quality of plant compounds was applied to analyze the chemical composition of *Crocus* perianth and its extracts. Sample pretreatment conditions and HPLC chromatography conditions were first optimized by investigating the effect of extraction solvents, extraction times and methods on extraction efficiency, as well as the influence of the mobile phase and detection wavelength on the efficiency of chromatographic separation of marker compounds. The optimal extraction and chromatographic conditions for *Crocus* used in this study can be found in the literature [39]. A typical HPLC chromatogram with a satisfactory resolution of 16 chemical markers is shown in Figure 5. The purity of the peaks was determined by comparing the retention times and UV spectra. The combination of a fingerprint with the quantification of analytes is more informative and can be obtained simultaneously. Of the 16 standards used, only 12 were found in the studied samples, and 4 compounds were additionally detected by the recalculation method. In *Crocus* raw material, flavonoids rutin (160.45 mg/g), apigenin-7-*O*-glucoside (8.11 mg/g), and isoorientin (7.39 mg/g) were found in the largest amount. Furthermore, mangiferin, isoquercitrin, rutin ferulic acid, as well as isoflavones (tectoridin, nigricin, iristectorignin B) were firstly identified in *C. sativus* perianth from the Ukraine.

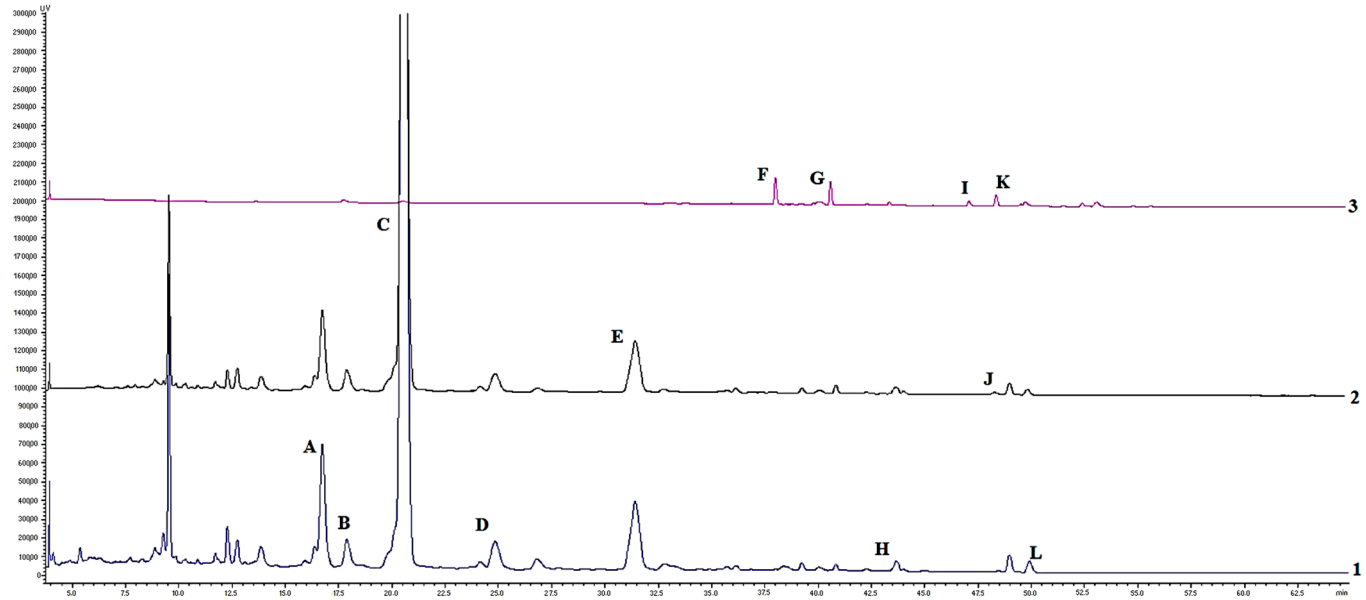

Figure 5. The chromatogram of *C. sativus* perianth fingerprints obtained by HPLC-DAD method at 270 nm (line 1), 310 nm (line 2), and 440 nm (line 3): mangiferin (**A**), isoorienthin (**B**), rutin (**C**), ferulic acid (**D**), isoquercitrin (**E**), tectoridin (**F**), crocin 4 (**G**), apigenin-7-glucoside (**H**), quercetin (**I**), nigricin (**J**), iristectorignin B (**K**), kaempferol (**L**).

The HPLC method was validated for parameters such as linearity range, LoD, LoQ, accuracy, precision, repeatability (Table S1), and specificity for each analyte. The calibration curve equations of standards along with the significance, LoD, and LoQ values, are presented in Table 3. The regression equation and correlation coefficient ranging from 0.9994 to 0.9999 revealed a good linearity response within the tested ranges. The series of calibration solutions were prepared and separated under the optimal conditions as described above. The LoD and LoQ values indicate that the proposed method demonstrates good sensitivity for the quantitative determination of 16 phenolic compounds in *Crocus* perianth. The repeatability was observed in the range from 0.23–1.06%, which was satisfactory and indicated the good repeatability of the proposed method. The determination of the main compounds in the test solutions was carried out by comparing the retention times of peaks and the UV spectrum obtained from the chromatogram of the standard solution (Table S2). All results showed repeatability, accuracy, high sensitivity, and good linearity of the method.

Table 3. Calibration curves of the reference standard compounds.

	Compound	Calibration Curve [a]	Correlation Coefficient r^2 (n = 6)	Linear Range (μg/mL)	RSD, %	LoD [b] (ng/mL)	LoQ [c] (ng/mL)
1	Mangiferin	f(x) = 29,263.5x + 13,863.9	0.999795	0.28–145.00	1.32	310	940
2	Isoorientin	f(x) = 26,559.9x + 2849.65	0.999996	0.73–92.85	1.41	8	24
3	Ferulic acid	f(x) = 54,955.4x − 638.345	0.999959	0.44–56.50	1.60	30	80
4	Rutin	f(x) = 16,072.5x + 1499.73	0.999879	0.16–20.24	1.07	96	290
5	Isoquercitrin	f(x) = 24,139.7x + 3904.44	0.999894	0.35–44.56	1.02	73	220
6	Crocin	f(x) = 3789.03x + 220.836	0.999588	1.15–147.20	1.28	100	300
7	Tectoridin	f(x) = 76,104.4x + 114,152	0.999580	0.51–260.00	0.55	130	400
8	Astragalin	f(x) = 20,536.0x + 1618.68	0.999987	0.37–47.70	1.01	90	270
9	Apigenin-7-glucoside	f(x) = 38,477.5x + 4025.41	0.999925	0.25–32.00	0.82	53	160
10	Quercetin	f(x) = 39,349.5x + 1454.47	0.999850	0.16–20.08	0.67	31	90
11	Kaempferol	f(x) = 29,888.8x + 1814.27	0.999924	0.14–18.32	0.90	37	110
12	Iristectorigenin B	f(x) = 109,562x + 68,062.7	0.999681	0.23–120.00	0.85	50	150
13	Nigricin	f(x) = 89,415.4x + 103,288	0.999404	0.35–181.00	0.30	40	130
14	Safranal	f(x) = 39,230.1x – 11,887.2	0.999529	1.33–42.56	1.35	120	360
15	Caffeic acid	f(x) = 57,646.8x − 3853.48	0.999922	0.72–91.92	1.56	20	60
16	Apigenin	f(x) = 50,138.3x + 5722.97	0.999889	0.2–25.76	0.53	25	80

[a] concentration of compound (mg/mL); y, peak area; [b] LOD, limit of detection (S/N = 3); [c] LOQ, limit of quantification (S/N = 10).

2.5.2. Perianth Extracts

Considering that the composition of *Crocus* active metabolites differs depending on the extractant used, ethanol and hydroethanolic extracts from perianth were obtained (Table 4). All peaks in the chromatograms were identified by comparison with the chromatogram of the mixed reference solution at three wavelengths of 270, 310, and 440 nm. At 440 nm the main saffron component, crocins, were determined. Crocins, as hydrophilic carotenoids, were better extracted in the water extract with an amount of 3.79 mg/g, whereas in the hydroethanolic extract it was only 0.2 mg/g. According to Montoro et al. [10], crocins were also detected in *C. sativus* perianths by the LC-ESI-MS method in positive ion mode.

Table 4. Compound content (mg/g) in *C. sativus* perianth and its crude extracts.

Compound	Retention Time, min/λ, nm	Raw Material	*Crocus* Perianth Extracts	
			Water	Hydroethanolic
Mangiferin	13.83/270	1.060 ± 0.751	1.091 ± 0.014	0.885 ± 0.010
Isoorientin	16.74/310	7.389 ± 0.369	0.668 ± 0.112	-
Kaempherol-3-*O*-sophoroside	17.94/310	2.329 ± 0.114	-	-
Rutin	20.46/310	160.45 ± 7.805	81.157 ± 0.580	65.785 ± 1.089
Ferulic acid	22.74/310	0.025 ± 0.08	0.045 ± 0.004	0.247 ± 0.003
Isoquercitrin	24.76/350	2.704 ± 0.104	1.785 ± 0.004	1.322 ± 0.026
Tectoridin	31.34/270	2.230 ± 0.093	1.423 ± 0.003	0.921 ± 0.070
Apigenin-7-*O*-glucoside	31.41/340	8.114 ± 0.387	2.587 ± 1.587	-
trans-Crocin 4	38.00/440	2.662 ± 0.113	3.788 ± 0.015	0.203 ± 0.003
trans-Crocin 2	40.59/440	2.299 ± 0.109	-	-
Quercetin	43.63/310	0.481 ± 0.019	0.229 ± 0.540	0.253 ± 0.003
cis-Crocin 4	47.07/440	0.591 ± 0.028	-	-
cis-Crocin 3	48.36/440	1.356 ± 0.057	0.809 ± 0.361	-
Nigricin	48.94/270	0.117 ± 0.015	0.052 ± 1.137	0.099 ± 0.022
Iristectorigenin B	49.15/270	0.142 ± 0.05	0.139 ± 0.012	0.142 ± 0.006
Kaempferol	49.43/310	0.916 ± 0.031	0.820 ± 0.003	1.018 ± 0.021

- = Not detected; values are presented as mean ± standard deviation from triplicate investigations. Statistical comparisons were performed using ANOVA test ($p < 0.05$).

Mangiferin was found in both extracts of *Crocus* perianths. The mangiferin content was higher in the water extract (1.09 mg/g) than in the hydroethanolic extract (0.89 mg/g), due to the presence of a glycoside residue. It is known that plants of the *Iris* genus (Iridaceae) accumulate various xanthones, mainly C-glycosylxanthones [38]. In the *Crocus* genus, mangiferin was identified (color reactions) only in *C. aureus* and *C. stellaris* leaves [33]. Therefore, mangiferin can be considered as a marker of the family. Taking into account the large mass of perianth waste, this type of raw material can be considered as an alternative to obtaining an individual mangiferin compound with potential antivirus activity.

Previous reports showed the presence of kaempferol and some quercetin glycosides, isoorientin, isoqueritrin, and astragalin in *C. sativus* tepals extract [7,50]. Flavonoid glycosides are better extracted by water, which we can observe for apigenin-7-*O*-glucoside and isoorientin (flavone C-glycoside of luteolin), which were identified only in the water extract of the perianth, 2.59 mg/g, 4.76 mg/g, and 0.67 mg/g, respectively. In addition, a similar pattern was observed for other identified flavonoid glycosides. For rutin (quercetin-3-*O*-rutinoside), a significantly higher content was found for the water extract of *Crocus* perianth (81.16 mg/g) than for the hydroethanolic extract (65.79 mg/g). It should be noted that rutin was first identified in *C. sativus* [7,10]. Isoqurcitrin (quercetin 3-*O*-glucoside) and quercetin were found in both *Crocus* perianth extracts. The content of isoquercetrin in the hydroethanolic extract was less (1.32 mg/g) than in the water extract (1.78 mg/g), while the content of quercetin in the extracts was approximately the same (0.2 mg/g).

Isoflavones have been identified in *C. sativus* for the first time. In the perianth water extract, the content of tectoridin (7-glucoside tectorigenin) was higher (1.42 mg/g) compared to the hydroethanolic extract (0.92 mg/g). Iristectorigenin B was found in the perianth in an approximately equal amount as the extracts (0.14 mg/g). The content of nigricin in the hydroethanolic extract was higher (0.10 mg/g) than in water (0.05 mg/g), the solubility and extractivity of the compound are due to the presence of the 2-OCH_3 group in the molecule, which makes it difficult to dissolve in water. Thus, the influence of substituents on the solubility of compounds is once again confirmed by the example of the dependence of the content on the structure. Previously, tectoridin and iristectorigenin B were isolated or identified only in the *Iris* plants genus [38]. This is the first report of the identification of those compounds in *C. sativus* perianth. In addition, new compounds identified in *Crocus* perianth extracts are nigricin, mangiferin, and rutin. Only a small amount of ferulic acid,

which is a biosynthetic precursor of O-phenylpropanoids in plants, were found in the perianth extracts.

By comparing the content of all compounds in the perianth extracts, it can be seen that the yield of the components in the water extract is higher since glycoside derivatives are more identified. Thus, the obtained results of the chemical composition and content of biologically active compounds of *C. sativus* has led to pharmacological studies of its extracts.

Among the constituents, the amount of rutin in the raw material of *C. sativus* perianth is high (>160 mg/g), therefore, it was selected as a Q-marker (ranking score 5). After structure–activity analysis and evaluation of the Herb MaRS criteria, five main compounds, including crocins, rutin, isoquercitrin, ferulic acid, and mangiferin, were selected based on their use for the treatment of different cancer cell lines (Figure 6). This ranking scale takes into account the clinical and pharmacological uses of the compounds and their claimed indications.

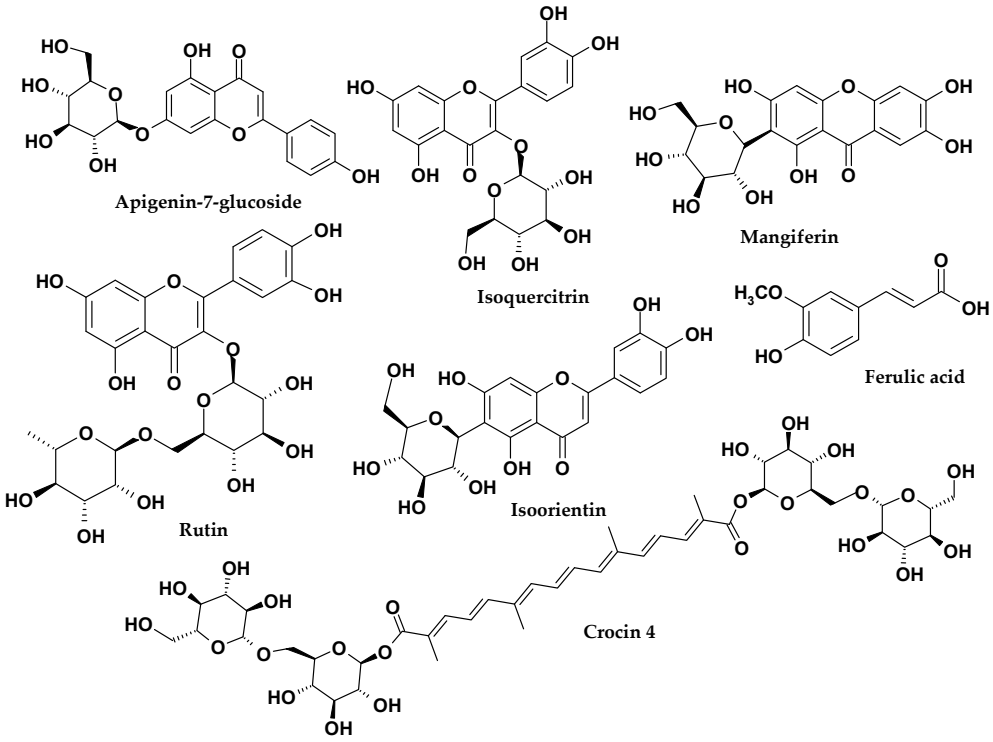

Figure 6. Chemical structure of selected quality markers and some major compounds of *C. sativus* perianth.

2.6. Assessment of Crocus Perianth Crude Extract Bioactivity

2.6.1. Antioxidant Activity

Nine compounds with antioxidant properties were identified in *Crocus* perianth extracts including mangiferin, isoorientin, ferulic acids, rutin, apigenin-7-glucoside, iristectorigenin B, and nigricin using the ABTS post-column assay. The antioxidant activity of the identified compounds was assessed by comparing their activity to the Trolox standard and expressed as TEAC values (mmol/g) and presented in Table 5. The perianth hydroethanolic extract showed higher activity (400.86 mmol/g) than Trolox (385.5 mmol/g). In the case of ABTS, stronger antioxidant activity was observed in *Crocus* perianth hydroethanolic extracts for mangiferin (128.13 mmol/g) and quercetin (121.11 mmol/g). *Crocus* raw material has shown potential bioactivity and antioxidant activity related to its radical scavenging capacity.

Table 5. The radical scavenging activity of individual compounds expressed as TEAC (mmol/g) between *C. sativus* perianth extracts using the ABTS post-column assay.

Compound	Retention Time	Water Extract	Hydroethanolic Extract
Mangiferin	15.34	15.76 ± 0.28	128.13 ± 2.25
Isoorientin	18.67	5.51 ± 0.10	-
Rutin	22.42	7.78 ± 0.14	3.77 ± 0.07
Ferulic acid	23.77	9.12 ± 0.16	110.15 ± 1.94
Tectoridin	29.90	15.16 ± 0.27	11.60 ± 0.20
Quercetin	44.37	10.05 ± 0.18	121.11 ± 2.13
Apigenin-7-*O*-glucoside	46.82	6.13 ± 0.11	-
Iristectorigenin B	51.71	13.47 ± 0.24	23.06 ± 0.41
Nigricin	52.88	5.66 ± 0.10	3.04 ± 0.05
Total		88.64 ± 1.56	400.86 ± 7.05

- = No activity in ABTS post-column assay. Trolox equivalent (0.3995 µmol/g) antioxidant capacity (TEAC) was used to express antioxidant activity of *Crocus* extracts; values are presented as mean ± standard deviation from triplicate investigations. Statistical comparisons were performed using ANOVA test ($p < 0.05$).

2.6.2. Cytotoxic Activity of Extracts

C. sativus perianth water and hydroethanolic extracts reduced the viability of melanoma (IGR39) (EC_{50} 0.50 and 0.58 mg/mL, respectively) and triple-negative breast cancer (MDA-MB-231) (EC_{50} 1.25 and 1.20 mg/mL, respectively) cell lines (Figure 7) via in vitro assay. Both extracts were approximately twice more active against the melanoma IGR39 than the breast cancer MDA-MB-231 cell line. The ethanolic extracts from the perianth were slightly more active than the water extract against the melanoma cells line. The *Crocus* perianth water extract was more active against the melanoma than the breast cancer cell line.

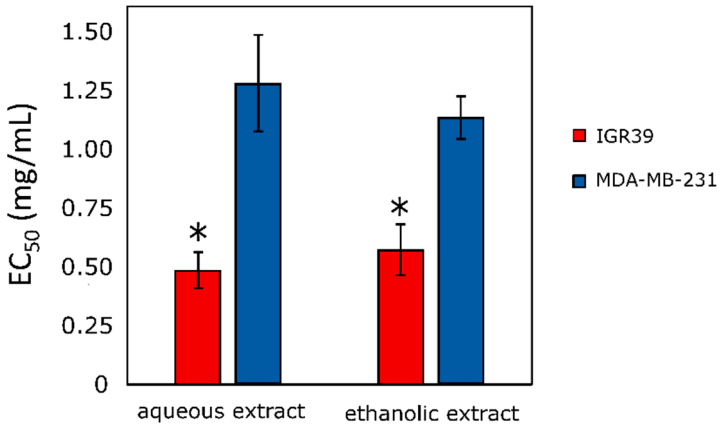

Figure 7. EC_{50} values of *C. sativus* perianth extracts against MDA-MB-231 and IGR39 cell lines after 72 h, * $p < 0.05$, $n = 3$.

The activity of the water extract of *Crocus* perianth is higher for the melanoma IGR39, probably due to the high content of glycosides, which is also similar to some of the authors' studies [85,109]. Some published data showed that glycosylated flavonoids exhibit more pronounced effects (anticancer, antidiabetic, antistress, antiallergic, antidegranulating, anti-inflammatory) than their aglycones [110]. However, there is a lack in vivo studies to confirm or disprove these findings.

3. Materials and Methods

3.1. Plant Material

C. sativus L. perianth was collected from the farm "Crocus.pro" (Ukraine) in November 2021. The saffron field was located in Borochyche village, Horokhiv district, Volyn region, Ukraine (50°25′46″ N; 24°49′25″ E) at an altitude of 222 m a.s.l. Stigmas were separated from flowers and dried for 2–3 h at 36 °C in a forced-air oven. Dried stigmas and flowers were stored in dark glass jars at 4 °C until analysis was performed. The specimen was deposited

at the Herbarium of V.M. Karazin Kharkiv National University, Ukraine (CWN, voucher specimen No. CWN0056541). The plant cultivation and primary process were in accordance with the WHO Guidelines on Good Agricultural and Collection Practices (GACP) [25]. Following procedures established by ISO 3632 1, 2:2010–2011, moisture content and the amount of picrocrocin, crocins, and safranal for *Crocus* stigma were determined to identify the sample quality category.

3.2. Quality Characterization of Crocus Perianth

The powdered materials of *Crocus* perianth (0.1 g, 60 mesh) or crude extracts were weighed into a volumetric flask, and methanol (10 mL) was used for extraction. The flask was placed in an ultrasonic bath at room temperature (20 ± 2 °C) for 30 min. The solutions were filtered through a membrane filter (0.45 µm) into glass vials. An aliquot of 10 µL was injected twice into the HPLC system for analysis. The reference compounds were used to prepare the standard solutions at a concentration of 1.0 mg/mL in methanol and used for calibration. The samples were stored at 4 °C before use.

3.3. Extraction Procedure of Crocus Perianth for Bioassay

Crocus perianth was dried, ground, and the powder was extracted with distilled water in a water bath at 100 °C (100 g, 1 L, 60 min × 3) or 70% ethanol at room temperature (100 g, 1 L, 60 min × 3). The extracts were concentrated to dryness.

3.4. Condition of HPLC and HPLC-ABTS Analysis

Detailed conditions of the component analysis of the plant samples by HPLC are described in previous works [39]. A brief description is given as follows: compound separations were performed in an ACE C_{18} column (250 mm × 4.6 mm, 5.0 µm; Zorbax Eclipse Plus, Agilent, Santa Clara, CA, USA). The flow rate was 1 mL/min. The solvent system included solvent A (0.1% acetic acid in water) and solvent B (acetonitrile). An ultrasonic bath was used for degassing, then all solvents were filtered using a filter with a 0.22 µm membrane. A linear gradient program was applied: 0 min–95% A and 5% B, 7 min–95% A and 5% B, 67 min–0% A and 100% B, 69 min–95% A and 5% B, 75 min–95% A and 5% B. The column temperature was kept constant at 25 °C. The injection volume of the sample solution was adjusted to 10 µL. Chromatographic peak identification was carried out according to the analyte and reference compound retention time by comparing the UV absorption spectra of the reference compounds and analytes obtained with a diode array detector. A quantitative method for the compound determination has been reported in previous work [39]. The detector wavelength was set to 270 nm, 310 nm, and 440 nm. HPLC-ABTS analysis was performed using a Waters Alliance 2695 separation module system with some modifications. The standard Trolox antioxidant (0.3995 µmol/g) was used for the preparation of the calibration curve. Trolox equivalent antioxidant capacity (TEAC) was used to express antioxidant activity [111]. Validation of the HPLC method was performed according to the guidelines ICH Q2 (R1) "Validation of analytical procedures" [112] by the following parameters: LoD, LoQ, specificity, linearity, and precision (Tables S1 and S2). Details are described in the Supplementary Materials section.

3.5. In Vitro Assessment of Cytotoxic Activity

The potential cytotoxic effect of *Crocus* perianth extracts against melanoma (IGR39) and triple-negative breast cancer (MDA-MB-231) cell lines was determined by an MTT viability assay as described before [39]. Details can be found in the Supplementary Materials section.

3.6. Experimental Design

According to the guideline ICH Q8 "Pharmaceutical Development" [5], the QbD conception is based on a clear definition of the aim of the experiment, planning, and control at each stage of the entire process of obtaining plant extracts from *Crocus* perianth. The

Design of Experiments (DoE) was used to design the design space. The experimental design included the selection of research objects, reagents, and extractants, parameters of extraction processes, chromatography conditions, and primary pharmacological screening. To reduce the number of experiments, a final screening design was used to investigate the effect of these parameters on the manufacturing process. Many process parameters can also be studied in very few experiments.

3.7. Risk Analysis

Risk assessment consists of identifying and describing potential hazards, assessing exposure, and characterizing the risk. An Ishikawa diagram analysis was performed by the guideline ICH Q9 "Quality Risk Management" [27], to identify factors that could affect the extraction of compounds from *Crocus* raw material. Failure Mode and Effect Analysis (FMEA) is proposed as an indispensable tool for classifying risks based on severity (S), probability of occurrence (O), and probability of detection (D) of raw materials at risk. In the FMEA, risk in the final product is expressed in terms of RPN (risk priority number), which is defined as follows: RPN = S × O × D. If RPN > 130, corrective action should be taken. FMEA considers any element that is part of the entire system [29]. The analysis of all possible reasons why each component or subsystem may not perform its intended function is carried out based on both the best opinion of experts and historical information on similar elements. The FMEA analysis is built based on the evaluation of hazardous treatments and the calculation of RPN, as shown in Table 1 and Table S3, and corrective actions are proposed for each identified hazard.

3.8. Herb MaRS Approach

To determine the most appropriate chemical markers for the quality control of *C. sativus* perianth and extracts, the Herbal Chemical Marker Ranking System (Herb MaRS) was used (developed by the National Institute of Complementary Medicine (NICM) at the University of Western Sydney, 2014 [17]). The Herb MaRS method takes into account various factors associated with herbal ingredients, such as the availability of biological activity studies and purely chemical reference standards; the relationship of the traditional or current use of the herb to its therapeutic use or pharmacological effects; the concentration of the chemical marker in the herbal product and the toxicity or maximum recommended dose. The Herb MaRS criteria contain a priority list of chemical markers, rationally ranked on a scale from 0 to 5, with 5 indicating the most appropriate chemical marker and rank 0 denoting least suitable. In addition, the "X" category indicates that there were no studies on the biological activity of the compound available at the time of selection. These compounds cannot be completely ruled out as potential chemical markers due to unknown activity.

3.9. Data Analysis

All data processing was carried out using the LabSolutions Analysis Data System (Shimadzu Corporation). Phenolic compound content and Trolox equivalent antioxidant activity of phenolic compounds was expressed as mean ± standard error (SE), $n = 3$. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with the software package Prism v.5.04 (GraphPad Software Inc., La Jolla, CA, USA). The value of $p < 0.05$ was taken as the significance level. The data were processed using the Microsoft Office Excel 2010 (Microsoft, JAV) software package.

4. Conclusions

It is now especially important to develop and apply standards that guarantee the quality, safety, and efficacy of herbal drugs. The design of experimentation and the selection of adequate chemical markers for quality control purposes require a good knowledge of the chemical composition of medicinal plants and their associated biological properties. We applied the Herb MaRS criteria to prioritize the selection of chemical markers for

quality control for *C. sativus* perianth, whilst also taking into account cancer bioactivity and its concentration in the extract. Crocins, flavonoid glycosides (rutin, isoquercitrin), xanthone mangiferin, and ferulic acid have been proposed as potential chemical markers for the quality control of *Crocus* perianth and its crude extracts. *C. sativus* perianth has shown potent antioxidant and anticancer bioactivities that might be related to the radical scavenging capacity of its major components. Thus, further pharmacological tests of *Crocus* raw materials as anticancer raw materials have scientific justification and prospects. In addition, the presence of high-quality stigmas (I category according to ISO 3632) will determine the availability of the raw material base of *Crocus* perianth. Implementing the Quality by Design approach will assist in managing the risks for quality control and processes in herbal drugs.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm90010019/s1, Tables S1–S3; Figures S1–S5. Materials and Methods.

Author Contributions: Methodology, Data curation, Writing—original draft, O.M.; Formal analysis, Investigation, I.B.; Conceptualization, Resources, Visualization, L.I.; Methodology, Investigation, V.P.; Conceptualization, Writing—review and editing, Supervision, V.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviation

ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
CPP	critical process parameters
CQAs	critical quality attributes
DoE	Design of experiment
EMA	European Medicines Agency
FDA	United States Food and Drug Administration
FMEA	Failure Modes and Effects Analysis
GACP	Good Agricultural and Collection Practices
GAP	Good Agricultural Practice
GHP	Good Handling Practice
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Points System
Herb MaRS	Herbal Chemical Marker Ranking System
HPLC-DAD	high-performance liquid chromatography coupled with diode array detector
ICH	International Council for the Harmonization of Specifications for Pharmaceutical Products for Human Use
QbD	Quality by Design
Q-marker	quality marker
QTPP	quality target product profile
WHO	World Health Organization

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