ORIGINAL RESEARCHES

https://doi.org/10.52418/moldovan-med-j.66-2.23.01
UDC: 615.322.074:[547.567.2+547.913]:582.949.27

Obtaining thymoquinone and thymohydroquinone from Wild bergamot (*Monarda fistulosa* L.)

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Manuscript received April 5, 2023; revised manuscript June 7, 2023; published online December 27, 2023

Abstract

Background: Thymoquinone (TQ) and thymohydroquinone (THQ) are natural substances with antibacterial, antimycotic, antiviral, anticancer, antioxidant activity. Previously, was developed the method for obtaining Wild bergamot essential oil with high TQ content. The objective of this study was to create methods for obtaining individual substances TQ and THQ from Wild bergamot herb.

Material and methods: Aerial parts of Wild bergamot have been collected from the plantation of the Scientific Practical Centre of *Nicolae Testemitanu* State University of Medicine and Pharmacy. The hydrodistillation method has been used for essential oil isolation and fractionation, and purification of individual substances. The analytical part of study has been performed by the HPLC method.

Results: THQ was obtained in crystalline form by reducing TQ directly in the essential oil with ascorbic acid, sulphurous acid, or natural components of the Wild bergamot essential oil. Subsequent oxidation of THQ with hydrogen peroxide or nitrous acid gave TQ. Additionally, the yield of TQ was increased by optimising the parameters of plant material fermentation and using two-stage hydrodistillation with intermediate oxidation of the THQ formed at the first stage.

Conclusions: Several technological procedures were created to obtain THQ from the Wild bergamot essential oil with a yield from 68-74% to 93-97% and TQ from THQ with a yield of 87-92%. The procedure of plant material processing has been optimised also to obtain essential oil with 48-56% TQ and not more than 12% residual phenols.

Key words: *Monarda fistulosa* L., essential oil, thymoquinone, thymohydroquinone.

Cite this article
Casian I, Casian A. Obtaining thymoquinone and thymohydroquinone from Wild bergamot (*Monarda fistulosa* L.). *Mold Med J*. 2023;66(2):5-11. https://doi.org/10.52418/moldovan-med-j.66-2.23.01.

Introduction

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone, TQ) and thymohydroquinone (2-isopropyl-5-methylhydroquinone, THQ) are phytochemicals found in several genera of plants of the *Lamiaceae* family, such as *Monarda*, *Thymus*, *Satureja*, as well as in the species *Nigella sativa* L. [1, 2].

TQ, studied as a pure substance or as the basic active principle of Black cumin oil (*Nigella sativa* L.), has demonstrated a wide spectrum of actions, including hepatoprotective [3, 4], antioxidant [5-7], antibacterial [8, 9], antimycotic [10], anti-inflammatory [11], anticancer [12, 13], antituberculosis [14], antiviral [15, 16], spasmolytic [17]. The literature describes the obtaining of TQ by chemical synthesis [18, 19] and by isolation from plant material [20].

THQ, being a reduced form of TQ, has a higher antioxidant and prooxidant activity [5]. Antibacterial [8], antimycotic [10], anticancer [13], and antiviral [15] activities have also been found. At the same time, THQ remains less studied in terms of pharmacology.

The TQ content in the Wild bergamot essential oil, obtained by traditional methods, is relatively low, being dependent on drying and storage conditions, as well as on the technological parameters of the plant material processing [21]. The intensification of TQ accumulation in Wild bergamot oil (up to 10-40%) can be achieved by applying some agrotechnical procedures [22, 23]. Previously, was developed the method for obtaining Wild bergamot essential oil with high TQ content (20-32%) from pre-fermented plant material [21], which made it possible to carry out a study for isolation of individual substances TQ and THQ from this oil.

The objective of this study was to create laboratory methods for preparative obtaining individual substances

TQ and THQ from the Wild bergamot herb, and to optimise conditions of plant material processing for obtaining the essential oil most suitable for this purpose.

Material and methods

Plant material. Aerial parts of the Wild bergamot, free of lignified stems, have been collected from the plantation of the Scientific Practical Centre of Medicinal Plants of *Nicolae Testemitanu* State University of Medicine and Pharmacy. A part of the plant material was immediately processed to obtain the essential oil, the rest was air-dried for further processing.

Apparatus. Fermentation of previously humidified plant material has been performed in a cylindrical extractor with an internal volume of 4.5 L, installed in an air thermostat. The plant material temperature and oxygen concentration in the air, blowing through the extractor, were measured with DS18B20 digital thermometer (*Dallas Semiconductor*) and 4OXV oxygen sensor (*City Technology*) respectively, and registered during the entire process with a laboratory-made data logger based on RP2040 microcontroller (*Raspberry Pi Ltd*). Essential oil isolation has been performed by hydrodistillation, using a steam generator with electric power of 1.1 kW, the same extractor, where plant material was fermented, and a glass flow cooler. Analysis of plant material, essential oil, reaction mixes, and the obtained samples of individual substances has been performed using *Agilent 1260* liquid chromatograph with diode-array UV detector in conditions described previously [21].

Chemicals. In the study have been used reference substances: Thymol, Carvacrol, and TQ (*Sigma-Aldrich*). Ascorbic acid was of pharmaceutical grade (*Ph. Eur.*) THQ reference solution was obtained *in situ* as a product of TQ reduction by ascorbic acid. Other reagents of analytical grade have been purchased from *Sigma-Aldrich Chemie GmbH* and *Merck* (Germany).

Results and discussion

The direct isolation of TQ from Wild bergamot essential oil, based on the difference in its physical properties from other components, is notably difficult. Thus, according to the observations, crystallisation of TQ is possible when its content in the essential oil (or any fraction) is higher than 65-70%. Furthermore, the yield of TQ is very low, and its subsequent purification is difficult. The use of preparative chromatography is more efficient but highly expensive. The idea of solving this problem was prompted by the spontaneous crystallisation of THQ, observed during storage of essential oil obtained from dry Wild bergamot herb, as a result of the reduction of TQ, contained in it, with some natural components [21]. The relatively high polarity of THQ causes its low solubility in essential oil, and its non-volatility facilitates its purification from volatile components. Actually, the idea was to reduce quantitatively TQ into THQ with one of the suitable reducing agents, to separate crystalline THQ from the oil phase, and to remove foreign volatile components by distillation with water vapour. Then the obtained THQ can be reconverted to TQ with a suitable oxidising agent.

The solution to this problem required additional optimisation of the previously described technology [21] for obtaining Wild bergamot essential oil rich in TQ. The reason was that residual amounts of thymol and carvacrol increase the solubility of THQ in the reaction mixture (fig. 4), thereby reducing its yield. In this regard, the conditions for the plant material fermentation have been optimised in the direction of the most complete oxidation of volatile phenols into TQ, while avoiding significant wastage of their vapours with air. This was achieved by programming the temperature during the fermentation process, reducing the speed of blowing air, and increasing the overall duration of the process. Additionally, the parameters of hydrodistillation of the fermented plant material were optimised. Instead of blowing with a mixture of steam and air, was used two-stage distillation with clean steam at high speed with intermediate air oxidation. This made it possible to reduce the loss of essential oil during distillation and achieve a high yield of TQ, even when using an extractor with increased volume.

An optimised variant of plant material processing was implemented in laboratory conditions, as the following procedure:

0.45 kg of dried Wild bergamot herb was humectated, mixing and slightly crushing, with 0.6 L of water or hydrolate waste from the previous batch, then loaded into a cylindrical extractor of 4.5 L volume. The extractor was kept at room temperature for a day and at 30-35°C for the next 2-3 days, blowing with air at 0.05-0.07 L/min and changing the air direction to the opposite every day, to prevent drying the segment of the plant material closed to the inlet. The fermentation rate was appreciated by the oxygen consumption. A typical diagram of the outlet oxygen concentration is present in fig. 1. The lower temperature on the first day of fermentation reduces the loss of volatile phenols, allocated outside the peltate trichomes, especially if the plant material was humectated with hydrolate waste. At long-term (more than 2-3 months) storage of plant material, the activity of oxidative enzymes is significantly reduced. In this case, the total duration of the fermentation can be increased to 5-6 days.

The extractor with fermented plant material was wrapped with heat-insulating cloth, connected between the steam generator and flow cooler, and about 250 mL distillate was collected into a narrow-necked flask of 500 mL volume. Immediately after turning off the steam generator, the extractor was blown with air at 0.5 L/min for 3 hours to oxidise the THQ, formed during the first hydrodistillation, into TQ. A small amount of condensate, formed during this operation, was collected into the same flask. Then hydrodistillation was repeated in the same regime until the receiving flask was filled. From the narrow part of the flask with a pipette were taken 11-14 g of essential oil with

TQ content of 48-56% and the amount of residual phenols from 5 to 12%, depending on the activity of enzymes in the plant material. Hydrodistillation was continued until another 100 mL of condensate was obtained, which was combined with hydrolate waste from the main receiving flask and used to humidify the plant material in the next batch. Using waste hydrolate instead of water improves the wettability of the plant material and increases the yield of the essential oil and TQ by about 8-9%.

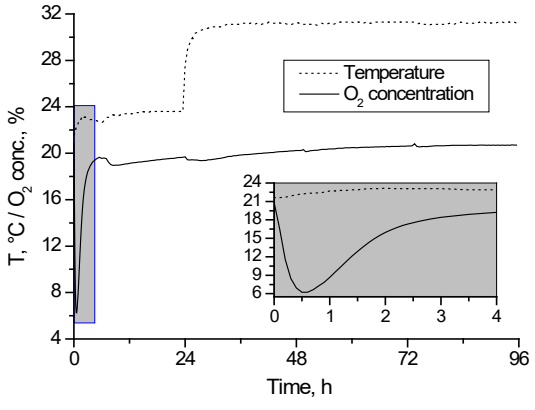

Fig. 1. Temperature and oxygen concentration change during the fermentation process

Obtaining thymohydroquinone. For this purpose, Wild bergamot essential oil with high TQ content has been treated with various reducing agents.

Method 1 – reduction of TQ with ascorbic acid: 20 mL of Wild bergamot oil rich in TQ and 10-20 mL of diluent were added to a 100 mL flat-bottom flask. Separately, a buffered solution of ascorbic acid was prepared as follows: Ascorbic acid in an amount of 1.29 g per gram of TQ, added with essential oil, (20% excess *vs.* stoichiometric amount) and sodium phosphate dodecahydrate in an amount of 0.2 g per gram of TQ was dissolved in 2.5 Mol/L sodium hydroxide solution, taken in an amount of 2.5 mL for each gram of TQ. The solution was cooled to room temperature, adjusted, if necessary, to pH 6.0-6.3 with sodium hydroxide or phosphoric acid, then completed with water to a volume approximately equal to the volume of the oil mixture (essential oil + diluent). The resulting solution was transferred into the flask with the oil mixture and intensively mixed with a magnetic stirrer for 1.5-2.5 hours. The end of the reaction was detected by the cessation of colour change of the reaction mixture and was confirmed by determination of the residual amount of TQ in the oil layer, which usually did not exceed 0.2%. The chemical reaction proceeds according to fig. 2.

THQ forms as a crystalline precipitate in the oil layer. Too low pH values of the aqueous layer slow the reaction, while too high values result in the formation of a fine precipitate of THQ and significant amounts of dark-coloured by-products.

Method 2 – reduction of TQ with sulphurous acid: In a 100 mL flat-bottom flask, 20 mL of Wild bergamot oil rich in TQ and 10-20 mL of diluent were added. Separately, potassium metabisulphite, taken in an amount of 0.75-0.81 g for each gram of TQ added with the Wild bergamot oil (10-20% excess), was dissolved in 30-40 mL of water. This solution was transferred into the flask with the oil mixture, and a small amount (0.5-0.7 mL) of 2.5 Mol/L sulphuric acid was introduced into the aqueous phase to initiate the reaction. Then a sufficient amount of sulphuric acid, necessary for the formation of sulphurous acid, is formed as one of the products (fig. 3). The contents of the flask were intensively mixed with a magnetic stirrer for 2-3 hours until the colour change ceased. As in the previous method, THQ is formed as a crystalline precipitate in the oil layer.

In both described methods, the primary role of the diluent is to reduce the viscosity of the oil layer during the formation of the THQ suspension and to allow efficient mixing of the layers. Its other function is to decrease the concentration of phenolic components of the essential oil, which promotes the crystallisation of THQ. Studies with different fractions of the oil layer, separated after finishing the reaction, and with their model mixtures showed that the solubility of THQ in the oil phase almost linearly depends on the total amount of thymol and carvacrol (fig. 4). This has a direct impact on the loss of the target product

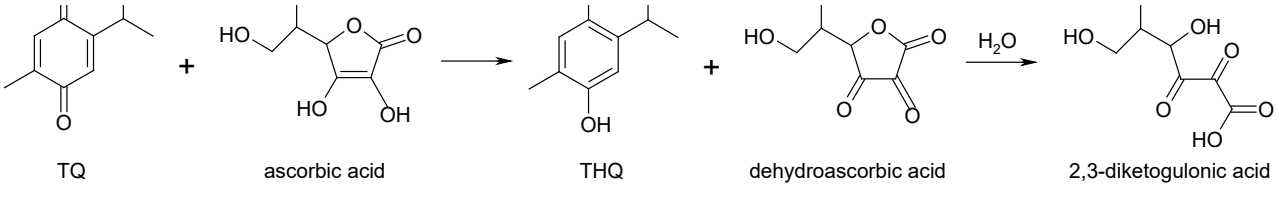

Fig. 2. Reduction of TQ with ascorbic acid

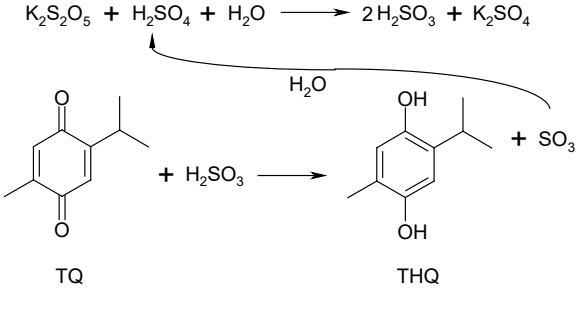

Fig. 3. Reduction of TQ with sulphurous acid

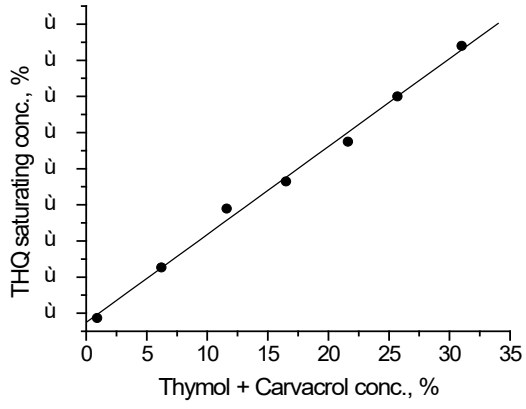

Fig. 4. Solubility of THQ in the oil phase in dependence on the phenols content

with the oil layer, and has become the main reason for optimising the conditions of plant material fermentation to minimise the residual amount of phenols in the essential oil.

Saturated hydrocarbons, such as n-hexane, can be used as a diluent. But, for environmental reasons, individual low-polar components of essential oils, such as pinene, limonene, p-cymene, as well as a light fraction of the waste oil layer of the reaction mixture from previous batches, are more suitable. The last option is the most attractive since it does not require additional solvents and reduces the amount of unused waste.

Waste fractionation was carried out by hydrodistillation according to the following procedure: 160 g of the waste oil layer from several experiments for the THQ obtaining and 0.8 L of water were distilled from a 2 L flat-bottom flask with a dephlegmator, collecting two distillate fractions. The first fraction with a volume of 250 mL was obtained at a heating power of 0.3 kW, and 95.4 g of light components mixture with a total phenol content of 0.9% was separated from it. The second fraction with a volume of 600 mL was distilled at a heating power of 0.5 kW, and 51.4 g of oil with 27.3% phenols was separated from it.

The presence of natural components with reducing properties in the composition of Wild bergamot essential oil is of particular interest. Unfortunately, these components are almost completely oxidised during the fermentation of plant material, but they are preserved in the essential oil obtained from fresh Wild bergamot herb. Their content is especially high in the light fraction of the essential oil. This fact led to the development of another way to obtain THQ. Since the chemical nature of the reducing components is currently unknown, the limited task was set to determine their total reducing power in reaction with TQ.

Method 3 – reduction of TQ with components of Wild bergamot essential oil: Wild bergamot essential oil rich in TQ and essential oil from the fresh herb or (preferably) its light fraction were mixed in a hermetic vial of suitable volume in a ratio, calculated from a predetermined value of reducing power. The vial was kept at 60°C for 3 days, then allowed to cool and left for 1-2 days at 4°C to complete the crystallisation of THQ, usually forming a monolithic crystalline mass, passing into suspension with mixing or shaking.

Fractionation of Wild bergamot essential oil to perform TQ reducing: 103 g of essential oil from fresh Wild bergamot herb and 0.3 L of water were distilled from a 2 L flat-bottom flask with a dephlegmator at a heating power of 0.28 kW, collecting two distillate fractions of 100 mL each. From the first fraction, 27.7 g of the oil layer was separated and used to reduce TQ. The second oil fraction (16.4 g) was similar in its composition to the initial oil and could be re-fractionated. The third oil fraction was separated from the vat residue in the amount of 54.0 g. It contained about 80% of phenols and can be studied as a potential active substance with antimicrobial properties.

Determination of reducing power: In a hermetic 2 mL vial for HPLC samples, 0.5 mL of Wild bergamot essential oil rich in TQ and 0.5 mL of the essential oil from fresh Wild bergamot herb or 0.3 mL of its light fraction were mixed. The vial was thermostated at 60°C for 24 hours, then the concentration of TQ in its content was determined in parallel in the initial Wild bergamot oil. The required volumetric ratio of the two types of essential oil was calculated using the formula:

$V_X = V_1/V_0 * C_0/(C_0 - K * C_1)$, where $K = (V_0 + V_1)/V_0$.

Definitions:

V_X – the volume of the oil from fresh Wild bergamot herb or its light fraction (in mL), required to reduce TQ in 1 mL of the oil from fermented plant material;

C_0 – the concentration of TQ in the initial oil from the fermented plant material;

C_1 – the concentration of TQ in the mixture after the test experiment;

V_1 and V_0 are, respectively, the volumes of oil from fresh Wild bergamot herb, or its light fraction, and the oil from fermented plant material, taken in the test experiment.

Separation and purification of THQ. In all three methods, the THQ precipitate was separated from the liquid phase by filter centrifugation, followed by triple washing with small amounts of water. The last operation allows the removal of water-soluble products of the chemical reaction (for methods 1 and 2) and a part of the oil phase. To remove the residues of the essential oil, the wet precipitate of THQ was transferred to a flat-bottom flask of sufficient volume and placed on a heating magnetic stirrer. Water was added in an amount of 15 mL per gram of TQ in the composition of used essential oil, and ascorbic acid in an amount of about 10 mg per gram of TQ, to protect THQ from oxidation. Then, with continuous stirring, at least half of the added amount of water was evaporated. The flask was allowed to cool slowly and kept until the next day at 4°C to complete the crystallisation. The precipitate was separated on a glass filter under a weak vacuum, washed with cooled 0.1% acetic acid solution, and dried to a constant weight at 40°C.

The resulting product is an odourless white crystalline powder, with a melting point of 142-142.5°C, easily soluble in ethanol and acetonitrile, slightly soluble in chloroform, very slightly soluble in water, and practically insoluble in hexane. The substance contains about 98% THQ, and the main impurities are related hydroquinone derivatives formed by the reduction of corresponding benzoquinones from Wild bergamot essential oil.

Reduction of TQ with ascorbic acid (method 1) can be recommended as the most efficient (93-97% yield of the target product) and safe enough way to obtain THQ.

Method 2 (reduction with sulphurous acid) is the cheapest. However, was observed a significant slowdown of the reaction toward the end of the process, especially under a slight excess of the reducing agent. As a result, it was not possible to achieve complete consumption of TQ. At the same time, a significant excess of sulphurous acid at the end of the reaction greatly increases the solubility of THQ in the aqueous phase and its loss with the aqueous layer waste. As a result, the final yield of THQ was only 68-74%. Another disadvantage of this method is the emission of toxic sulphur dioxide into the atmosphere.

Reduction of TQ with natural components of the same plant (method 3) is very attractive in sense of personal and environmental safety since it does not involve the use of any chemicals. The yield of THQ is quite good (about 82%). However, the need to obtain additionally the essential oil from the fresh plant material and the long duration of the procedure, carried out at an increased temperature, make this method the most expensive.

Obtaining thymoquinone can be performed by oxidation of THQ with various oxidising agents. The main problem was to achieve a sufficiently high rate and completeness of the reaction with a minimum amount of by-products. In this study, were used two oxidising agents: hydrogen peroxide and nitrous acid, which have their advantages and disadvantages.

Method 1 – oxidation of THQ with hydrogen peroxide in aqueous suspension: Into a 250 mL flat-bottom flask were added 15 g of ground and sieved THQ, 0.15 g of sodium lauryl sulphate (for better wetting of THQ), 75 mL of water, and 15 mL of concentrated hydrochloric acid. The mixture was sonicated and cooled to 4°C. Then 1.5 mL of 1 Mol/L copper (II) chloride solution and 59 mL (15% excess) of chilled 6% hydrogen peroxide were added at stirring. The mixture was stirred for 2 hours, cooling the flask with flowing water, and then kept at 4°C for the next 2 hours. The precipitate was separated on a glass filter and washed with chilled water.

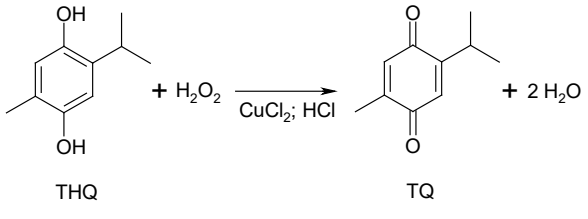

Fig. 5. THQ oxidation with hydrogen peroxide

The main chemical reaction proceeds according to the scheme in fig. 5.

Copper (II) chloride is used as a catalyst. Actually, the catalytic activity belongs to the anionic complex $[CuCl_4]^{2-}$ formed in the presence of hydrochloric acid. Cooling the reaction mixture and slow addition of hydrogen peroxide contribute to the formation of a smaller amount of by-products.

Purification of TQ: The wet precipitate of crude TQ was transferred to a 2 L flat-bottom flask, 700 mL of water was added, and about 500 mL of liquid was distilled without a dephlegmator at a heating power of 0.5 kW, maintaining the condensate temperature within 47-55°C to prevent crystallisation of TQ in the cooler. The distillate was kept until the next day at 4°C, crystalline TQ was separated on a glass filter, washed with chilled water, and dried at 35°C. 13.2 g of purified product were obtained (about 88% yield).

It is also possible to obtain thymoquinhydrone (TQH) by introducing in the reaction exactly half of the stoichiometric amount of hydrogen peroxide, then separating and drying the precipitate without further purification. TQH is obtained as a black-coloured crystalline powder and presents a hydrogen bond complex of equimolar amounts of TQ and THQ. It melts in the range of 88-95°C with decomposition into its constituent components. It is easily soluble in ethanol and acetonitrile, sparingly soluble in chloroform, and slightly soluble in water. It is partially soluble in hexane with decomposition.

Method 2 – oxidation of THQ with hydrogen peroxide in aqueous-acetonitrile solution: 15 g of THQ was added to a 250 mL flat-bottom flask, dissolved in a mixture of 60 mL acetonitrile and 15 mL of concentrated hydrochloric acid. The following operations were performed in an ice bath. 1.5 mL of 1 Mol/L copper (II) chloride solution was added, then, with continuous stirring, 56 mL (10% excess) of 6% hydrogen peroxide solution was injected with a peristaltic pump at 1.5 mL/min. Stirring was continued for another 1 hour (until the liquid turned yellow). The reaction mixture was saturated with 15 g of sodium chloride and allowed to stand to separate the layers. TQ is contained in the acetonitrile (upper) layer.

Isolation and purification of TQ: The upper liquid layer (57 mL) was transferred to a 2 L flat-bottom flask, 120 mL of water was added, and acetonitrile was distilled off with a dephlegmator at a heating power of 0.25 kW up to the vapour temperature of 94°C. The distillate (46 mL), containing about 83% acetonitrile and about 0.5% TQ, can be used in the next batch instead of a part of acetonitrile.

600 mL of water was added to the residue in the flask and about 500 mL of liquid was distilled without a dephlegmator at a heating power of 0.5 kW, maintaining the temperature of the condensate within 47-55°C to prevent crystallisation of TQ in the cooler. The distillate was kept until the next day at 4°C, crystalline TQ was separated on a glass

filter, washed with chilled water, and dried at 35°C. 13.1 g of the purified product was obtained (about 87% yield).

Method 3 – oxidation of THQ with nitrous acid: In a 100 mL flat-bottom flask, 15 g of THQ was added and dissolved in a mixture of 60 mL acetonitrile and 20 mL of concentrated hydrochloric acid (10% excess). With continuous stirring, 42 mL of 30% sodium nitrite solution (about 1% excess) was injected with a peristaltic pump at 0.7 mL/min (until finishing the intensive elimination of nitric oxide, which has been decomposed in the flame of a gas burner). Alternatively, the nitric oxide can be absorbed with a potassium permanganate solution. Stirring was continued for another 10 min. The mixture was allowed to stand for layers' separation, and the upper layer, containing TQ, was taken. In this case, saturation with sodium chloride is not required, since its sufficient amount is formed in the chemical reaction (fig. 6).

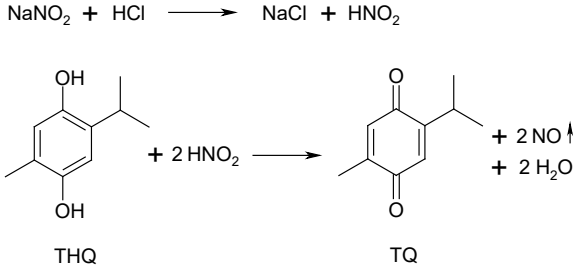

Fig. 6. THQ oxidation with nitrous acid

Isolation and purification of TQ were carried out the same way as described in method 2. In this experiment 13.8 g of the purified product was obtained (about 92% yield).

The obtained TQ is an orange-yellow crystalline powder or conglomerates of crystals with a slight odour. Its vapours are irritant to the mucous membranes of the eyes and respiratory tract. The melting point of TQ is 45-46°C. It is very easily soluble in acetonitrile and chloroform, freely soluble in ethanol and hexane, and very slightly soluble in water. The substance contains about 98% of TQ, and the main impurities are related substances of the benzoquinone structure.

Method 1 is the most environmentally friendly since it is not associated with organic solvents or highly toxic substances. At the same time, passing the reaction in a suspension leads to reduced robustness of the process and an increased amount of by-products. The situation is complicated by the poor wettability of THQ in aqueous solutions and its tendency to float. The last problem is solved by adding a surfactant (sodium lauryl sulphate), but its excess increases the loss of TQ with the filtrate of the reaction mixture and can lead to the formation of a fine precipitate that is difficult to filter.

Method 2 gives well-reproducible results, but has a slightly higher labour intensity and requires an organic solvent (acetonitrile).

Method 3 is characterised by the fastest and most selective passing of the chemical reaction and, as a result, by the formation of the smallest amount of by-products. However, its serious disadvantage is the release of highly toxic nitric oxide, which must be captured and rendered harmless.

Conclusions

Several technological procedures were created to obtain thymohydroquinone from the Wild bergamot essential oil with a yield from 68-74% to 93-97% and thymoquinone from thymohydroquinone with a yield of 87-92%. The procedure of plant material processing has been optimised also to obtain essential oil with 48-56% of thymoquinone and not more than 12% of residual phenols.

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IC designed the study, conducted the laboratory work and performed its technological part, interpreted the data, and drafted the first version of the manuscript. AC collected and processed the plant material, performed the analytical part of the laboratory work, and revised the manuscript. The final version of the manuscript was approved by both authors.

Funding
This study was a part of the project No 20.80009.8007.14 "Complex researchers for the elaboration of the new autochthonous anti-infectious pharmaceutical products for the optimisation of the pharmacotherapy of the dental, oropharyngeal, and auricular disorders" within the State Program of the Republic of Moldova (2020-2023).

Ethics approval and consent to participate
No approval was required for this study.

Conflict of interests
No competing interests were disclosed.