















The Use of Ultrasonics for Stable Emulsion Preparation of Fir and Cedar Essential Oils

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Abstract: The properties of fir and cedar essential oils are very diverse. The main ones are antibacterial, anti-inflammatory, antifungal and antiviral. Furthermore, fir and cedar essential oils help to speed up metabolism and reduce stress. Currently, a lot of research is being done on the anti-cancer properties of these oils. The developed nanoemulsion makes it possible to use all the valuable substances of fir and cedar essential oils. Products with it can be used to prevent neoplasms in the human body and improve the functioning of the muscular system and individual organs, such as the liver. The interest in ultrasonic processing is based on the beneficial properties it brings to food products, particularly, disinfection, emulsification, intensification of some technological processes, and so on. Various creams, suspensions and emulsions are produced using ultrasonic processing. All over the world, research is underway to obtain and study the properties of these solutions. In this study, a technology for obtaining a stable nanoemulsion (oil-water) using ultrasonic vibrations has been developed. The results showed that the nanoemulsion with using Tween 80 emulsifier is the most stable. The rational ratio of oil: emulsifier: water was determined. The optimal surfactant for obtaining a stable emulsion is the TWEEN 80 emulsifier at a concentration twice the concentration of oils - the ratio of emulsifier to oil is 1:2, respectively. The appearance and formula of the emulsion obtained by ultrasonic generator is much better than that obtained by dispersion and mixing. To obtain a stable emulsion, treatment with an ultrasonic generator at a frequency of 22 kHz, power of 100% and processing in an ice bath is necessary. A comparative analysis of the appearance and stability of the obtained nanoemulsion was carried out in comparison with nanoemulsions obtained by other methods. This nanoemulsion can be used in the food industry to improve the antibacterial activity in food products, prolong the shelf life, and increase the nutritional value. Moreover, developed nanoemulsion can be widely used in the cosmetic and pharmaceutical industries for both oral preparations and transdermal use.

Introduction

Generators and ultrasonic equipment make it possible to mix "immiscible" liquids, such as oil and water, and obtain colloidal solutions, suspensions and emulsions. All over the world, research is underway to obtain and study the properties of these solutions. Essential oils have been highly valued and widely used since ancient times, in countries such as Persia, Egypt, China and India, but research and discovery of new oils continue to this day. Their most popular area of application is aromatherapy,

although there are many other uses for these oils. One of the growing industries that use the beneficial properties of oils is the food industry (Burt, 2004; Carson et al., 2006).

Essential oils are natural products derived from aromatic plants. Essential oils can be used in foods to extend shelf life and reduce or replace synthetic additives. Their effectiveness can be confirmed by antimicrobial and antioxidant tests (Wińska et al., 2019). Presently, they are mainly studied for their various



biological properties such as antioxidant, antimicrobial, antitumor, analgesic, insecticidal, antidiabetic and anti-inflammatory (Baitukalov et al., 2004; Reyes-Jurado et al., 2020; De Temmerman et al., 2011). In general, essential oils increase the stability of foods during storage by inhibiting the growth of spoilage and pathogens and by protecting against oxidation (Koul et al., 2008). Essential oils are widely used in medicine, perfumery, cosmetics and as food preservatives. In the 19th century, essential oils were used as medicines due to their aroma and taste. To date, 3,000 essential oils have been identified, of which about 300 types are used in perfumery due to their strong fragrance (Reische et al., 2008). There is growing worldwide interest in the multiform potential of natural products in combating foodborne pathogens using essential oils, especially of plant origin ones (Cheng, 2003). In food products, essential oils have shown antimicrobial and antioxidant activity, which can increase the shelf life of the product and ensure its quality (Maurya et al., 2005). In addition, essential oils have been well known as an alternative to reducing or replacing the use of synthetic additives, which are associated with various adverse human health effects (Baitukalov et al., 2004). While essential oils have proven to be a promising alternative to chemical preservatives, they have particular limitations that must be addressed before they can be used in food systems. Low water solubility, high volatility and strong odor are the main properties that make it difficult to use in food (Mediratta et al., 2002).

Fir (*Abies*) is a genus of 48-56 species of symmetrical, evergreen, coniferous trees belonging to the pine family. Fir essential oil is often obtained from the needles of the *Abies balsamea* or *Abies alba* (Mei et al., 2021) tree species. The largest producers of fir essential oil are France, Germany, and Bulgaria (Mei et al., 2021). Fir essential oil has numerous biological effects, including antibacterial (Song et al., 2022), antifungal, anti-mite and anti-mosquito properties (Lemnaru (Popa) et al., 2023; Nedorostova et al., 2009; Nuutila et al., 2003). Due to its excellent antibacterial and antifungal properties, this oil can be used as a natural food preservative (Otero et al., 2014). Studies of anti-diabetic plants indicate the ability of fir (*Abies balsamea* (L.)) to increase glucose transport in muscle cells and adipocytes (Pandey et al., 2017). Also, this essential oil has a pronounced effect on glucose homeostasis in liver cells (Pandey et al., 2014).

Cedarwood essential oil is widely used in fragrances, as well as in scented soaps, aerosols and sprays, disinfectants, for cleaning microscope parts and

immersion lenses (Paniwnyk, 2017). The essential oil of cedar has antifungal, antimicrobial (Hawkins et al., 2022; Prosekov et al., 2018; Ramadass et al., 2019; Tamer et al., 2022), antiviral, molluscicidal properties (Huang et al., 2021; Perdones et al., 2016), and also exhibits anti-inflammatory activity (Pandey et al., 2016; Poaty et al., 2015; Taghavi et al., 2023). Have been shown that *Escherichia coli*, *Bacillus subtilis* and *Bacillus cereus* are susceptible to cedarwood essential oil (Pandey et al., 2016; Kačániová et al., 2022). The benefits of pine nut oil for acne treatment have been tested by measuring its bacterial activity against *Propionibacterium acnes* (Paniwnyk, 2017). Cedar essential oil demonstrates potent anti-cancer activity in animal and human cancer cells in vitro, as evidenced by studies on the effect of the oil on human myeloid leukemia cells (Roldan-Cruz et al., 2016).

While creating an emulsion, it is necessary to make a choice from hundreds and thousands of emulsifying agents (Wang et al., 2023). From this set, researchers select one or two that satisfactorily emulsify the ingredients chosen to create the emulsion (Sanders et al., 2002). Emulsion stability is achieved when surfactant molecules cover the entire oil/water interface, improving interfacial rheology and preventing droplets from sticking together and aggregating due to steric repulsion.

The three methods most commonly used to prepare emulsions are sonication, high-pressure homogenization and microfluidization (Sivakumar et al., 2014). The formation and size of droplets mainly depend on the amplitude of the ultrasonic power and the treatment time. Drop deformation resistance also depends on the surface activity of the emulsifier and its concentration in the continuous phase (Ye et al., 2013; Zhang et al., 2016; Zhang et al., 2020). To date, studies comparing ultrasonic emulsification with dispersion have shown that ultrasound is competitive or even superior in terms of droplet size and energy efficiency. Microfluidization has been found to be more effective than ultrasound, but less practical in terms of production costs, equipment contamination, and aseptic handling. Comparing mechanical agitation with sonication, it was found that for a given desired diameter, the amount of surfactant needed was reduced, energy consumption (due to heat loss) was lower, and ultrasonic emulsions were less polydisperse and more stable (Zrira & Ghanmi, 2016).

This work demonstrates the effects of ultrasonic treatment on obtaining a stable emulsion of fir and cedar essential oils in water. Various methods of introducing components and preparing an emulsion were considered,

and the stability of emulsions with different concentrations of surfactant was also investigated.

Materials and Methods

The following materials were used to prepare an oil-water emulsion of essential oils:

- Essential oil of fir (Mirrolla LLC, Russia);
- Essential oil of cedar (Mirrolla LLC, Russia);
- Emulsifier Polyethylene glycol 400 (PEG) (JSC NIZHNEKAMSKNEFTEKHIM, Russia);
- Emulsifier TWEEN-80 (Unitop Chemicals Pvt. Ltd., Mumbai);
- Distilled water.

TWEEN 80 emulsifier in different concentrations. The concentration of essential oils in all samples was 10% - fir essential oil to cedar essential oil in a 1:1 ratio. Some of the samples were processed with an Ultra-Turrax® laboratory disperser (IKA T 25, Germany) at 17,500 rpm for 5 minutes at a temperature of 25°C. Half of the dispersed samples were processed using an ultrasonic generator (Ultrasound Technology - INLAB LLC, Russia) at a frequency of 22 kHz and a power of 100% for 5 minutes. The samples without dispersion were stirred with a magnetic stirrer (IKA RCT basic, Germany) at 400 rpm for 10 minutes to obtain a short-

Table 1. Treatment samples preparation using an ultrasonic bath.

N	Emulsifier	Emulsifier concentration, % (v/v)	Oilmixture: emulsifier (v/v)	Ultrasonic processing
1	Polyethylene glycol 400	5	1:0.5	no
2				yes
3		10	1:1	no
4				yes
5		20	1:2	no
6				yes
7		30	1:3	no
8				yes
9	TWEEN 80	5	1:0.5	no
10				yes
11		10	1:1	no
12				yes
13		20	1:2	no
14				yes
15		30	1:3	no
16				yes

Treatment samples preparation using ultrasonic bath

To study the effect of treatment in an ultrasonic bath, 16 samples were prepared with two types of emulsifiers at different concentrations. The concentration of essential oils in all samples was 10% - fir essential oil to cedar essential oil in a ratio of 1:1(v/v). All samples were treated with a laboratory dispersant at 17,500 rpm for 5 minutes at 25°C. The samples subjected to sonochemical treatment were placed in an ultrasonic bath (Grad-Technology LLC, Russia) for 40 min at a frequency of 35 kHz and a power of 40%. Treatment at a shorter time and device power did not give the desired visual results and was excluded from the experiment. A longer treatment time and an increase in power are not advisable from the point of view of energy efficiency and the duration of the sample production process.

Treatment samples preparation using ultrasonic generator

To study the effect of sonochemical treatment using an ultrasonic generator, 12 samples were prepared with

term stable solution, and also treated with ultrasound at 22 kHz and 100% power for 5 minutes. Longer treatment time did not show any improvement in the result. During ultrasonic treatment, the samples were cooled with ice to remove the heat generated in the process.

The microscopy of the samples was carried out using a laboratory microscope Axio Lab.A1 (Carl Zeiss, Germany). To obtain microscopic pictures, emulsion samples were placed on a glass slide, then covered with a cover slip. After placing the slide on the microscope stage, an image was obtained with x63 magnification.

The emulsion stability of treatment samples of emulsion was determined experimentally by the measurement of the creaming index (CI) (Equation 1) in 24 h after manufacture and during the storage period every 5 days. An emulsion (35 mL) was placed into a 50 mL centrifugal plastic tube and centrifuged at 2,500 rpm for 15 min at 25°C. An emulsion (30 mL) was placed into a 50 mL centrifugal plastic tube and centrifuged at 7,500 rpm for 15 min at 25°C. The CI

Table 2. Treatment samples preparation using ultrasonic generator.

N	Emulsifier concentration, % (v/v)	Oil mixer: emulsifier (v/v)	Dispersion using laboratory dispersant Ultra-Turrax® (IKA T 25, Germany)	Mixing using magnetic stirrer (IKA RCT basic, Germany)	Ultrasonic processing using ultrasonic generator (Ultrasonic Technique – INLAB LLC, Russia)
1	5	1:0.5	yes	no	no
2			yes	no	yes
3			no	yes	yes
4	10	1:1	yes	no	no
5			yes	no	yes
6			no	yes	yes
7	20	1:2	yes	no	no
8			yes	no	yes
9			no	yes	yes
10	30	1:3	yes	no	no
11			yes	no	yes
12			no	yes	yes

values were obtained from the ratio between the total height of the cream layer (CC) and the total height of emulsion layer (TE).

$$CI(\%) = \frac{CC}{TE} * 100(1)$$

All experiments were performed at least in triplicate. Data were processed by methods of mathematical statistics at theoretical frequency of 0.95. Statistical processing of data was carried out using computer programs LibreOffice Calc 2021 and Mathcad 15.0.

Results and Discussion

The microscopic pictures of samples with 5% emulsifier concentration show that TWEEN 80 emulsifier stabilizes the emulsion better since the size of the fat globules is significantly smaller than in the samples with Polyethylene glycol 400.

The microscopic images of samples with 10% emulsifier concentration show that TWEEN 80 emulsifier with increasing concentration reduces the size of the fat globules of the resulting emulsion. Samples with PEG emulsifier also have smaller beads compared to samples with this surfactant at a lower concentration. However, they are larger than the samples with TWEEN 80.

Samples with a concentration of TWEEN 80 emulsifier 20% and 30% after sonication were visually evaluated: after treatment, the colour changed from white to greenish. This indicates a fairly small particle size.

During microscopy of all samples, the average value of the size of fat globules was calculated. Based on the obtained values, a graph of the dependence of the size of fat globules on the concentration of the emulsifier and the presence or absence of ultrasonic treatment was plotted. TWEEN 80 emulsifier shows the positive effect on fat

globule size compared to PEG surfactant. Ultrasonication also has a positive effect on particle size. The size of fat globules with ultrasonication is smaller than with dispersion without sonochemical treatment.

To study the stability of the emulsion, the samples prepared for the experiment were stored at a temperature of 25°C for 24 hours. The results obtained showed that it makes no sense to store the samples longer since a clear separation into the oil and water phases was found in the samples. Figure 5 shows samples with an emulsifier concentration of 20% and 30%, respectively, after ultrasonic treatment. Samples with a concentration of 5% and 10% dissolved after 2 hours of storage, which indicates their instability.

According to the visual assessment, the emulsion samples prepared using an ultrasonic bath are unstable after 24 hours of storage and cannot be further investigated. Apparently, the separation of the emulsion during storage occurred due to insufficient power, frequency, and area of impact of the ultrasonic bath on the samples.

Thus, treatment with an ultrasonic bath does not allow obtaining a storage-stable "oil-in-water" type emulsion of fir and cedar essential oils.

Samples prepared using dispersion and an ultrasonic generator were selected for further visual study. The experiment showed that the closer the consistency and color to the water, the smaller the particle size in the resulting samples.

When visually evaluating the obtained samples, there is a significant difference between the samples with an emulsifier concentration of 20% and 30% - these samples have a more transparent color compared to the rest of the

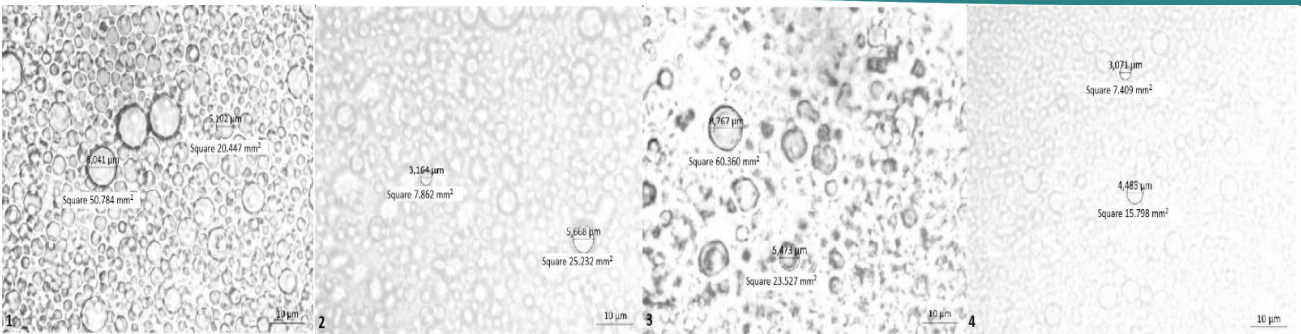


Figure 1. Microscopic images of emulsion samples with 5% emulsifier concentration: 1 - PEG without sonication, 2 - PEG with sonication, 3 - TWEEN 80 without sonication, 4 - TWEEN 80 with sonication.

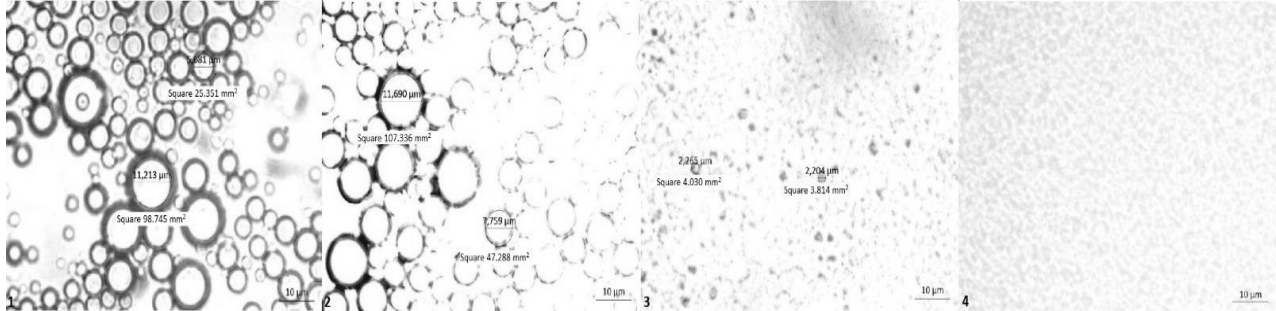


Figure 2. Microscopic images of emulsion samples with 10% emulsifier concentration: 1 - PEG without sonication, 2 - PEG with sonication, 3 - TWEEN 80 without sonication, 4 - TWEEN 80 with sonication.



Figure 3. Emulsion samples with emulsifier concentration of 20% and 30% after ultrasonic treatment: from left to right - PEG 20%, PEG 30%, TWEEN 80 20%, TWEEN 80 30%.

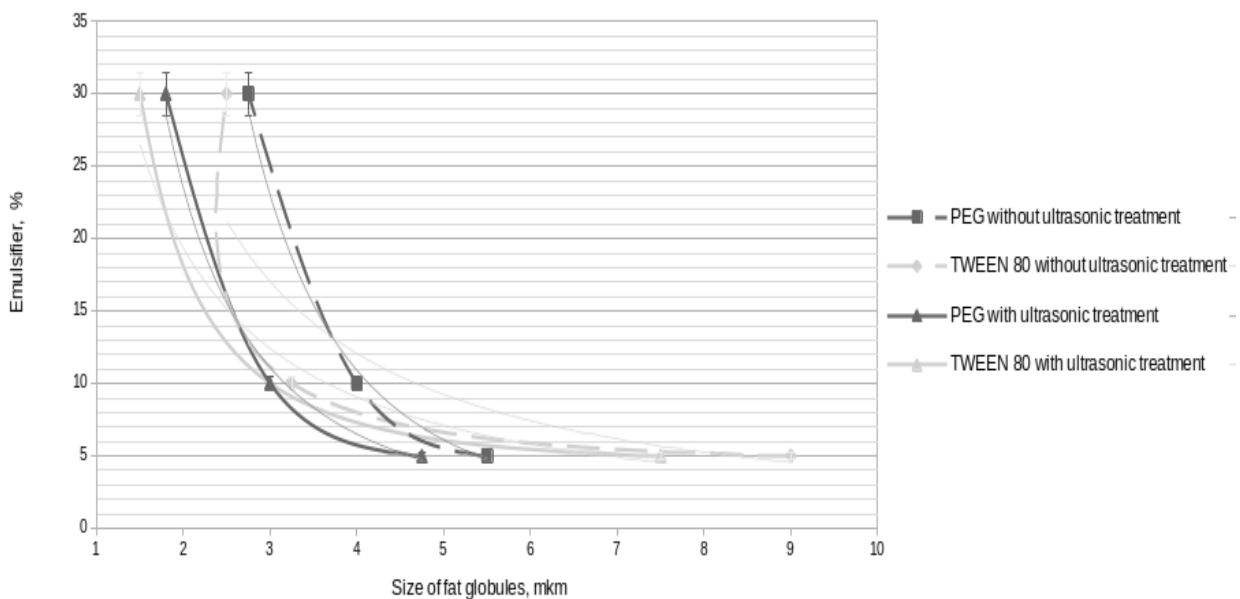
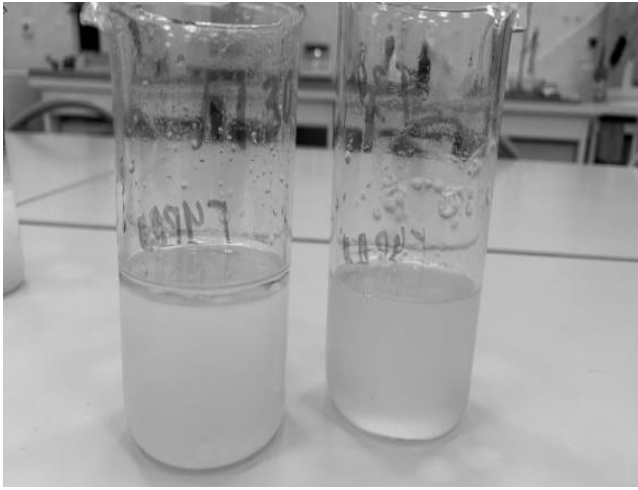
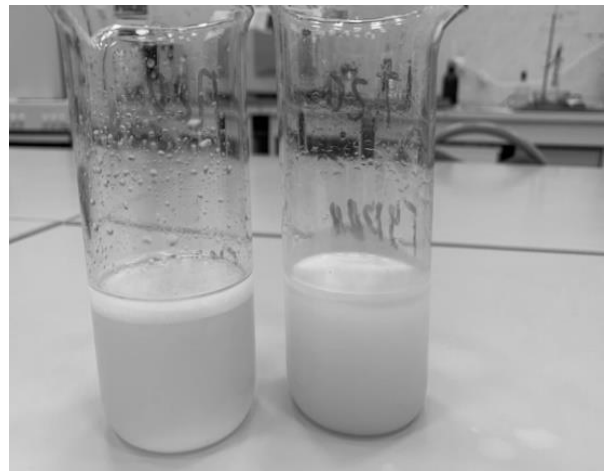


Figure 4. The dependence of the size of fat globules on the amount of emulsifier and the presence of ultrasound treatment.



Emulsifier concentration 20%



Emulsifier concentration 30%

Figure 5. Studied emulsion samples after ultrasonic treatment: on the left - PEG, on the right - TWEEN 80.



Dispersion at 17,500 rpm for 5 min



Dispersion at 17,500 rpm for 5 min and ultrasonic treatment at a frequency of 22 kHz and 100% power for 5 min



Mixing at 400 rpm for 10 min and ultrasonic treatment at a frequency of 22 kHz and 100% power for 5 min



Ultrasonic treatment at a frequency of 22 kHz and 100% power for 5 min

Figure 6. Prototypes with different concentrations of emulsifier and different cooking modes.

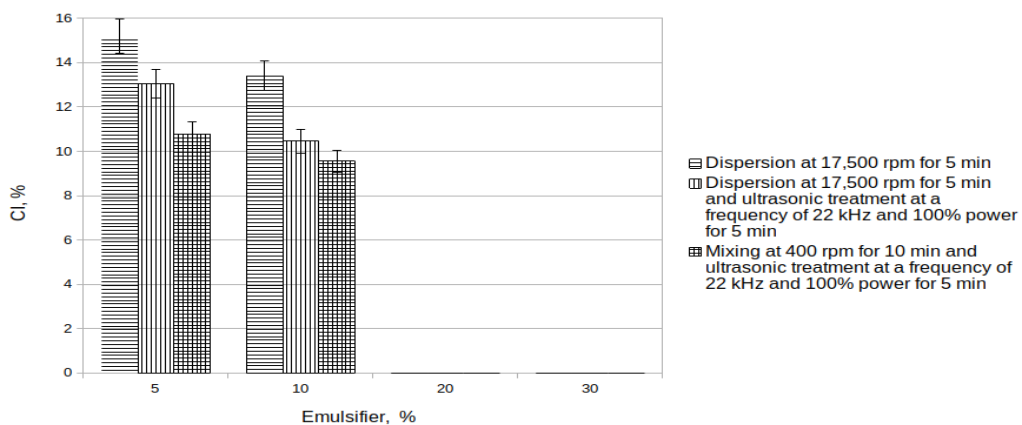


Figure 7. Stability of emulsion samples with different emulsifier concentrations and preparation method during storage.

samples. However, the samples after dispersion from foam. Foaming is an undesirable process when obtaining an emulsion.

Findings of a study demonstrate that with an increase in the concentration of the emulsifier, the value of the creaming index decreases, which indicates an improvement in the stability of the samples. Creaming index could not be measured in samples with 20% and 30% concentration, which indicates excellent storage stability. However, 30% of samples stored at 4°C have solidified, which may adversely affect the rheological properties of the product.

The appearance and stability of the emulsion processed by ultrasonic generator treatment are much better than those of the emulsion obtained by dispersion. To obtain a stable emulsion, the concentration of TWEEN 80 emulsifier should be twice the concentration of the oil phase, that is, the ratio of emulsifier to oil is 1:2 (v/v), respectively.

Conclusion

The presented work demonstrates that ultrasonic bath treatment does not allow obtaining a storage-stable oil-in-water emulsion of fir and cedar essential oils. The optimal surfactant for obtaining a stable emulsion is the TWEEN 80 emulsifier at a concentration twice the concentration of oils - the ratio of emulsifier to oil is 1:2, respectively. The appearance and formula of the emulsion obtained by the ultrasonic generator are much better than that obtained by dispersion and mixing. To obtain a stable emulsion, treatment with an ultrasonic generator at a frequency of 22 kHz, power of 100% and processing in an ice bath is necessary.

The obtained visual results and the demonstrated stability indicate fairly low small particles in emulsions, about 100 nm. This will be a further direction of research.

Products with such emulsion can be used for neurological prevention of neoplasms in the human body, improving the function of the muscular system and especially organs, such as the liver. The use of essential oils of fir and cedar improves the functioning of the nervous system of the body and reduces the risk of psychosomatic disorders. Developed emulsion can be used in the food industry to improve the antibacterial activity in food products, prolong the shelf life, and increase the nutritional value. Moreover, the emulsion can be widely used in the cosmetic and pharmaceutical industries, both for oral preparations and for transdermal use.

Conflict of Interest

The authors declare that there is no conflict of

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interest.

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