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Preparation of Aqueous Extracts of Biologically Active Substances from Tagetes Patula L. Flowers

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ABSTRACT:

The search for new sources of biologically active substances (BAS) and methods of obtaining safe antimicrobial plant preparations is a relevant direction of modern pharmacology and biotechnology. This study aimed to determine the most effective method for obtaining aqueous extracts of *Tagetes patula* L. flowers with antimicrobial properties. HPLC results showed that the highest content of BAS was characteristic for aqueous extracts obtained by boiling for 60 minutes (they contained the most luteolin and quercetin derivatives, quercetin itself, and protocatecholic acid, as well as rutin and ellagic acid). The maximum yield of gallic acid was observed in the extracts obtained by infusion with stirring. The aqueous extracts of *T. patula* flowers, obtained by boiling for 30 and 60 minutes, respectively, exhibited the greatest antimicrobial activity against both *E. coli* and *S. aureus*, while the diameter of the lysis zone increased in proportion to the duration of extraction, with the exception of the interval of 30-60 minutes for *E. coli*. The antimicrobial effect was more pronounced against *E. coli* than against *S. aureus*. Thus, *T. patula* aqueous extracts have potential applications for as medicines, natural pesticides, siderates, and feed additives.

Keywords: marigold; extraction; flavonoids; ultrasound; HPLC; antibacterial activity.

1. Introduction

Traditional herbal medicines have played an important role in human life and provided natural health benefits since antiquity (Shepel and Krieger, 2022). In modern times, however, synthetic drugs have replaced natural medicines, and the uncontrolled use of antibiotics has caused significant damage to human health, leading to pathogen resistance. In this context, the scientific community is actively developing safe health remedies from plant sources (Shepel and Krieger, 2023; Chaachouay and Zidane, 2024).

One of the most promising plants is *Tagetes patula* L. (French marigold), which has a long history of use in folk medicine and agriculture due to the presence of a wide range of useful properties. For example, marigolds can destroy and repel agricultural pests and control the growth and development of pathogens. Marigolds have the inherent ability to create favorable conditions for the growth of beneficial plants while suppressing the development of weeds (Tang *et al.*, 2024). Marigolds have significant insecticidal, fungicidal, and antibacterial properties and are an effective alternative to synthetic pesticides. In addition, marigolds are natural siderates that can structure and improve soil properties (Cerruti *et al.*, 2010), because the most important task in agronomy is the safe, renewable use of fertile land without the use of synthetic pesticides and fertilizers. In animal husbandry, preparations based on this plant can serve as an alternative to antibiotics used in feed additives to improve feed quality and safety (Wang *et al.*, 2024).

The relevance of this study is provided by the search and consideration of methods for extraction of biologically active compounds from *T. patula* flowers, as well as statistical analysis of the results obtained. The antimicrobial properties of samples of aqueous extracts of marigolds on *Staphylococcus aureus* and *Escherichia coli* and their potential applications as medicines, natural pesticides, siderates and feed additives were studied in detail.

This study aimed to determine the most effective method for obtaining samples of aqueous *Tagetes patula* L. flower extracts with antimicrobial properties.

2. Materials and Methods

Objects of research

The objects of the study at different stages of the work were samples of aqueous extracts of *Tagetes patula* L. flowers with hydromodule 1:10 obtained by different methods.

Preparation of aqueous extract samples from *T. patula* flowers

For each of the four methods, the following values are taken as constants: 1) hydromodule 1:10; 2) particle size: not more than 2 mm (the raw material was passed through a sieve with a mesh size of 2 mm); 3) 20 mL of water was added to 2 g of crushed raw material (exact suspension).

Then, extraction was performed using the following 4 methods, and the dynamics of the extraction process were considered (Table 1).

Table 1: Extraction methods used

No.	Extraction method	Parameters	Extraction time, min
1	Boiling on an electric stove	100 °C	5 - 60
2	Infusion with stirring on a heated magnetic stirrer	70 °C, 1000 rpm	5 - 60
3	Ultrasonic extraction	20 kHz, 95% amplitude, 130 W	5 - 20
4	Microwave extraction	2450 MHz, 700 W	0.5 - 2.0

The extraction modes used are summarized in Table 2.

Table 2: Values of time characteristic in different extraction methods

Extraction mode	Duration, min			
	Boiling	Infusion with stirring	Ultrasonic extraction	Microwave extraction
A	5	5	5	0.5
B	15	15	10	1.0
C	30	30	15	1.0
D	60	60	20	2.0

The obtained extract samples were filtered through a filter (Dia-M, Moscow, Russia), the microtube with the filtrate was centrifuged on a centrifuge (Puschinskie Laboratorii, Puschino, Russia) at 7000 rpm for 3 minutes to remove ballast substances.

Determination of the total phenolic compounds in aqueous extract samples of T. *patula* flowers

The amount of phenolic compounds was determined according to the following procedure (Ondua *et al*., 2019). The reaction mixture was prepared by adding 20 µL extract, 100 µL Folin-Ciocalteu reagent (10%) and 80 µL 7.5% aqueous Na_2CO_3 solution to the wells of a 96-well plate (BMG Labtech, Ortenberg, Germany). The solution mixture was placed in a dark place at room temperature for 30 minutes and the absorbance was measured at 765 nm on a BMG Labtech tablet reader (BMG Labtech, Ortenberg, Germany). The total concentration of phenolic compounds was calculated from the calibration curve obtained with gallic acid standard solution (100-4000 µg/mL). Data were expressed as mg of gallic acid equivalents per mL of extract.

Determination of the total flavonoid content in aqueous extract samples of *T. patula* flowers

The flavonoid content of the extracts was quantified using the method described in (Chookalaii *et al.*, 2020). A volume of 25 µL of extract, 100 µL of distilled water, and 7.5 µL of 5% sodium nitrite were transferred to the wells of a 96-well plate (BMG Labtech, Ortenberg, Germany). After 6 minutes, 7.5 µL of 10% aluminum chloride, 100 µL of 4% sodium hydroxide, and 10 µL of distilled water were added to each well. The plate was kept in the dark, and after 15 minutes, the absorbance of the solutions was measured using a BMG Labtech Tablet Reader (BMG Labtech, Ortenberg, Germany) at 510 nm. The same procedure was followed for the rutin standard solution to construct the calibration curve (100-4000 µg/mL). The flavonoid content of the extracts was expressed as mg of rutin equivalent per mL of extract.

Determination of the qualitative and quantitative composition of phenolic compounds and flavonoids in samples of *T. patula* flower extracts

The composition of phenolic compounds and flavonoids in samples of aqueous extracts of *T. patula* flowers with maximum retention time was determined by high-performance liquid chromatography (HPLC).

The analysis was performed on an LC-20AB Shimadzu Prominence chromatograph (Shimadzu, Kyoto, Japan) equipped with a binary pump and an SPD-M20A diode matrix detector. A Zorbax 300SB-C18 4.6*250 vv 5 µm column (Agilent, Santa Clara, CA, USA) was used for the determination. The separation was performed at 35 ℃ in gradient elution mode. Mobile phase: eluent A – 0.1% THF in bidistilled water, B – acetonitrile. Sample volume – 5 µL. Flow rate – 1 mL/minutes. Analytical wavelength – 254 nm.

Determination of the antibacterial activity of *T. patula* flower extract samples

Determination of antibacterial effect of all obtained samples of plant extracts was carried out

by standard disk-diffusion method. Pure bacterial cultures were used in the tests: *Staphylococcus aureus, Escherichia coli*. Determination of antibacterial effect of all obtained samples of plant extracts was carried out by standard disk-diffusion method. Pure bacterial cultures were used in the test. The medium was pre-sterilized at 1.5 atm for 15 min. For comparative evaluation of antimicrobial activity of plant extract samples, a disk with the following antibiotics was used as a positive control: clindamycin (2 µg) for *S. aureus*, amoxicillin/clavulanic acid (20/10 µg) for *E. coli*, and distilled water as a negative control. A swab was used to disperse the bacterial suspension onto solid media in Petri dishes in a continuous lawn technique over the entire agar surface until it was absorbed into the agar. The dishes were then placed in a thermostat with an internal temperature of 37 °C for 16 h. At the end of the incubation time, the dishes were removed from the thermostat and the diameter of the lysis zones around the disks was measured.

Methods of statistical analysis

Statistical processing was performed using IBM SPSS Statistics 27 program (IBM, Armonke, NY, USA). Measurements were performed in three repetitions and the mean ± standard deviation was found for the results obtained. Comparative analysis of the BAS content in the studied extracts was performed by means of one-factor and two-factor analyses using Shapiro-Wilk and Tukey's criteria (at the significance level of $p < 0.05$).

3. Results and discussion

Total content of phenolic compounds in samples of *T. patula* flower aqueous extracts and statistical processing of the results

Table 3 presents the average yields of phenolic compounds in the extracts.

Table 3: Average yield values of phenolic compounds in extract samples, mg/mL

Extraction mode	Extraction method			
	Boiling	Infusion with stirring	Ultrasonic extraction	Microwave extraction
A	0.239 ± 0.009	0.220 ± 0.008	0.162 ± 0.005	0.242 ± 0.005
B	0.258 ± 0.006	0.207 ± 0.007	0.184 ± 0.004	0.228 ± 0.007
C	0.271 ± 0.003	0.292 ± 0.007	0.167 ± 0.003	0.201 ± 0.006
D	0.311 ± 0.008	0.264 ± 0.008	0.217 ± 0.006	0.256 ± 0.004

The highest yield of phenolic compounds was obtained through boiling, with a maximum yield reached after 60 minutes and a subsequent increase. The lowest yield was observed in the case of ultrasonic extraction. However, ultrasonic extraction is considered an effective method for extracting BAS from medicinal raw materials; it is possible that the exposure amplitude was too high and destructive for some substances and the extraction time was incorrect.

Total flavonoid content in *T. patula* flower aqueous extract samples and statistical processing of the results

The results of flavonoid content in the obtained samples are presented in Table 4.

Table 4: Average values of flavonoid yield in extract samples, mg/mL

Extraction mode	Extraction method			
	Boiling	Infusion with stirring	Ultrasonic extraction	Microwave extraction
A	0.292 ± 0.006	0.199 ± 0.007	0.131 ± 0.007	0.185 ± 0.009
B	0.402 ± 0.005	0.293 ± 0.007	0.178 ± 0.006	0.252 ± 0.008
C	0.577 ± 0.007	0.348 ± 0.006	0.156 ± 0.008	0.274 ± 0.008

D	0.550 ± 0.005	0.392 ± 0.008	0.151 ± 0.008	0.291 ± 0.007

The highest yield of flavonoids was observed in the boiling extraction, with a maximum yield achieved within 30 minutes. The lowest yield was observed in the ultrasonic extraction.

Results of antibacterial activity determination of *T. patula* flower aqueous extract samples

In order to observe the dependence of the antibacterial activity on the time and method of extraction, all samples under investigation were considered. Table 5 presents the results of antimicrobial test against *S. aureus* and *E. coli*.

Table 5: Diameter of growth inhibition zones, mm

Culture	Extraction method	Extraction mode				Control 1	Control 2
		A	B	C	D		
S. aureus	BES*	11	13	16	18	34	-
	ISH	9	8	14	11	34	-
	US	-	7	-	8	34	-
	MW	10	9	8	10	34	-
E. coli	BES	14	18	22	19	29	-
	ISH	8	14	15	16	29	-
	US	-	10	9	8	29	-
	MW	8	12	14	15	29	-

Control 1 – positive control, Control 2 – negative control
BES – Boiling on an electric stove; ISH – Infusion with stirring on a heated magnetic stirrer; US – Ultrasonic extraction; MW – Microwave extraction.

As can be seen in Table 5, the most effective against both *S. aureus* and *E. coli* were the samples of extracts obtained by boiling, with an increase in the diameter of the lysis zone depending on the duration of extraction, except for the 30-60 minutes interval for *E. coli*. With this extraction method, the maximum lysis zone occurs at 60 minutes for *S. aureus* and 30 minutes for *E. coli*. Furthermore, an increasing trend of dependence of the antimicrobial effect on extraction time was observed for the extracts obtained by infusion with stirring on a magnetic stirrer with heating and microwave extraction against *E. coli*. They showed good antimicrobial activity against this strain as well as against *S. aureus*, where there was no such trend. This may be due to the fact that extracts with different times of preparation have different compositions of constituent components (BAS) that may be selectively active against a given strain. This calls for additional research to study the more detailed chemical composition of *T. patula* aqueous extracts in a time-dependent manner. The extracts obtained by ultrasonic extraction showed weak antimicrobial effect, which may be due to the low yield of biologically active substances after extraction.

It can also be noted that the extracts generally had a greater antimicrobial effect on *E. coli* than on *S. aureus*, probably due to the selective action of the BAS contained in the extracts against Gram-positive and Gram-negative bacteria (Mekvimol *et al*., 2020).

Thus, the samples of aqueous extracts of T. patula flowers obtained by boiling for 30 and 60 minutes, respectively, had the highest antimicrobial activity against *E. coli* and *S. aureus*. This may be due to their high content of phenolic compounds and flavonoids compared to other

extracts studied.

Samples of aqueous extracts obtained by boiling for 60 minutes were used for further studies.

Results of the qualitative and quantitative composition determination of phenolic compounds and flavonoids in T. patula extract samples by HPLC

Figure 1 presents the chromatogram of the aqueous extract obtained by boiling with a maximum retention time of 60 minutes. Based on the HPLC data, the content of phenolic compounds in this extract was calculated (Table 6).

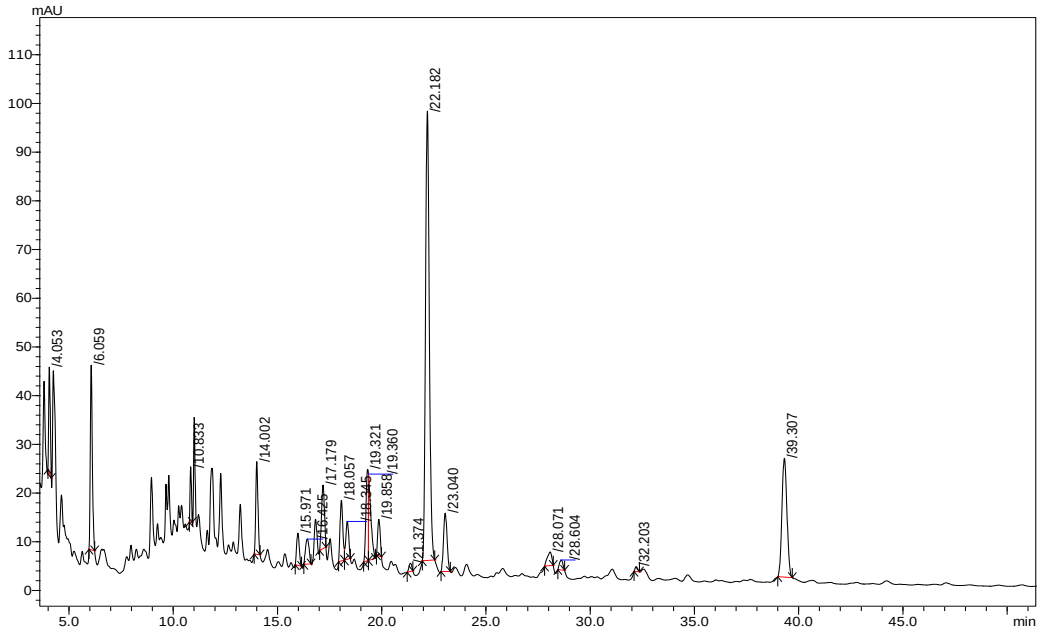

Figure 1: Chromatogram of the aqueous extract of Tagetes patula L. flowers obtained by boiling on an electric stove, 60 minutes, 1:10

Table 6: Concentration of phenolic compounds in *T. patula* flower aqueous extract samples obtained by boiling for 60 minutes, 1:10

Component	Retention time, min	Concentration, µg/mL
Gallic acid	4.2	8.4
Protocatechuic acid	6.0	9.6
Chlorogenic acid	10.8	4.8
Ellagic acid derivative	14.0	6.3
Ellagic acid	19.3	6.6

Five phenolic compounds were detected (Table 5, Figure 1) in samples of *T. patula* aqueous extract obtained by boiling for 60 minutes (1:10), the quantitative content of which decreases in the following order: protocatechuic acid (9.6 µg/mL), gallic acid (8.4 µg/mL), ellagic acid (6.6 µg/mL) and its derivative (6.3 µg/mL), chlorogenic acid (4.8 µg/mL).

Protocatechuic acid, found in higher amounts in this extract, has extensive pharmacological properties and may act as an antioxidant, anti-inflammatory, neuroprotective, anti-tumor, anti-diabetic, and anti-apoptotic agent. This acid may also be useful in the prevention and treatment of cancer, diabetes, Alzheimer's disease, atherosclerosis, and other diseases (Song *et al.*, 2020; Jalali *et al.*, 2020).

Ellagic acid is a potent anti-carcinogenic and anti-mutagenic compound involved in preventing the activation of environmental toxins, mutagens, and carcinogens. Finally, ellagic acid has antimicrobial properties, including against *Helicobacter pylori*, and can therefore be considered a promising antibacterial agent against *H. pylori*-associated gastroduodenal

diseases in humans (Vattem and Shetty, 2005).
The results of the HPLC determination of the flavonoid content in the samples of this extract are summarized in Table 7.

Table 7: Flavonoid concentration in T. patula flower aqueous extract samples (by boiling, 60 minutes, 1:10)

Component	Retention time, min	Concentration, µg/mL
rutin	19.9	3.9
quercetin	39.3	12.2
luteolin derivative	16.0	3.5
luteolin derivative	16.4	1.8
luteolin derivative	18.3	4.6
luteolin derivative	23.0	8.9
luteolin derivative	28.1	1.2
luteolin derivative	32.2	0.2
quercetin derivative	17.2	5.3
quercetin derivative	18.1	4.9
quercetin derivative	21.4	0.7
quercetin derivative	22.2	34.5

The data indicate that the aqueous extract of *T. patula* obtained by boiling for 60 minutes (1:10) contains significant quantities of luteolin and quercetin derivatives, as well as quercetin itself (12.2 µg/mL) and rutin (3.9 µg/mL) (Table 6).
Antioxidant treatment with quercetin may help prevent the toxic effects of mycotoxins in the food and feed industries. Quercetin has been shown to have neuroprotective effects, and its combined action with fish oil and ascorbic acid shows beneficial effects on neurodegenerative diseases (Yang *et al*., 2020; Batiha *et al*., 2020).
Rutin is known to have neuro- and cardioprotective, anticonvulsant, anti-ulcer, anti-asthmatic, hepato- and nephroprotective, anticancer, diuretic, antibacterial, antifungal, antiviral, wound healing, and radioprotective effects. This flavonoid is known for its ability to prevent blood clotting and strengthen blood vessel walls. It also has a sedative effect on the central nervous system, as well as antidepressant and analgesic properties (Ganeshpurkar and Saluja, 2017).

4. Conclusion

It was found that the most effective way to obtain aqueous extracts of *T. patula* flowers was boiling, which allowed to obtain the highest yield of phenolic compounds within 60 minutes, flavonoids – within 30 minutes.
In general, the extract samples had a greater antimicrobial effect on *E. coli* than on S. *aureus*, probably due to the selective action of the BAS contained in the extracts against Gram-positive and Gram-negative bacteria. According to the results of the antimicrobial test, aqueous extracts of *T. patula* flowers obtained by boiling for 30 and 60 minutes, respectively, had the highest antimicrobial activity against *E. coli* and *S. aureus*. This may be due to their high content of phenolic compounds and flavonoids compared to other extracts studied.
Further research is required to conduct a more volumetric and multidimensional analysis of various methods of aqueous extraction of BAS from *T. patula* flowers.

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Conflict of Interest declaration
The authors declare that they have NO affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Author Contributions
Olga Babich, Stanislav Sukhikh contributed to the design and implementation of the review, writing the original draft and editing; Ekaterina Shepel and Olga Kriger methodology, of the research,. All authors accepted the final draft.

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