

Article

Assessment of the Fungistatic Properties of *Calendula officinalis* L. Water Extract and the Effect of Its Addition on the Quality of Wheat Bread

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Abstract: The potential of *Calendula officinalis* water extract against fungi *Aspergillus niger* and *Penicillium* sp. and the effect of extract addition on the quality of wheat bread were investigated. In vitro, the extract reduced the mycelial growth and biomass production of *A. niger*, but there was no inhibitory effect on *Penicillium* sp. Enriched bread showed significantly higher total phenolic content, by about 77% and 95% in the bread, in which 10% and 15% of the water was replaced with extract, respectively. The antioxidant potential against DPPH• was significantly higher (compared to the control) in both variants used in the experiment, and the level of antioxidant activity increased with the addition of extract. The enriched bread had good quality characteristics—lower baking losses and higher volume than the control. The moisture content and acidity of the crumb of the extract-enriched bread were also higher. The extract additive used did not affect the sensory properties of the bread.

Keywords: *Aspergillus niger*; *Penicillium* sp.; marigold; biopreservatives; fungal control; bread fortification



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1. Introduction

Bread and other bakery products are important diet components in many parts of the world. The baking mainly uses white wheat flour obtained after removing wheat (*Triticum aestivum* L.) bran and germ, which are sources of fibre, phytochemicals, and important nutrients. Because of this, the final product contains less dietary fibre and phenolic compounds. Foods enriched with natural antioxidants are being developed to recompense for this loss. Functional foods consumed as part of a regular diet provide biologically active ingredients that provide health benefits [1]. Unfortunately, bread is a perishable food that goes through physical, chemical, sensory, and microbiological changes during storage. One of the biggest problems in baking is the growth of toxigenic fungi, mainly from the genera *Aspergillus* and *Penicillium* [2]. The growth of fungi leads to quality changes in food, but it can also have negative health effects, as some of these microorganisms produce mycotoxins. They are a public health problem due to their widespread occurrence in the world's food supply [3]. To avoid spoilage of food products by fungi, preservatives are used. However, they have some disadvantages, including altering the aftertaste of light flour products, delaying dough fermentation [4], and not being inert to the consumer. There are reports in the literature of the occurrence of urticaria and contact dermatitis after consuming products with sorbic acid, which is approved for preserving bread [5]. In addition, chemical preservatives are ineffective against some common fungal species in bread, such as *Penicillium paneum* and *P. roqueforti* [2].

The threat to food health safety posed by the presence of toxigenic fungi and the demand for natural products has prompted the search for new methods of preserving baked goods and eliminating harmful microorganisms and their metabolites. There is great interest in natural ingredients with multifunctional properties with potential uses in food production. Plant extracts have been widely studied as potential biopreservatives, as

plants contain many important antimicrobial compounds, including phenolic compounds, glucosinolates, alkaloids, cyanogenic glycosides, and oxylipins [6,7].

In this context, *Calendula officinalis* (common marigold), a member of the *Asteraceae* family, is a valuable product with rich medicinal and functional properties. Many secondary metabolites are responsible for the biological activity of marigolds, such as flavonoids, triterpenoids, and polyphenols, found mainly in flower extract [8,9]. These compounds are responsible for antioxidant activity, exhibit neuroprotective effects, prevent degenerative diseases such as diabetes and cardiovascular diseases, and have anticancer, anti-inflammatory, and antimicrobial effects [10]. Extracts from *Calendula officinalis* petals have proven antimicrobial activity against many pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus* [11], *Escherichia coli*, *Bacillus subtilis*, *Klebsiella aerogenes*, *Enterococcus faecalis*, *Candida albicans*, *C. glabrata*, *Aspergillus flavus*, *A. fumigatus*, and *A. niger* [12].

Reports from other researchers indicate that, depending on the plant species, the best solvents for extracting bioactive compounds are acetone, ethanol, and methanol [13,14]. However, according to current trends, analytical procedures should strive to be minimal, as simple as possible to perform, low-cost, and environmentally friendly. Therefore, the study presented in this paper decided to use deionized water as a solvent to extract bioactive compounds from marigolds. Water extract preparation is a relatively easy and inexpensive process, which is important from the consumer's point of view.

This study aimed to evaluate the fungistatic properties of the *Calendula officinalis* water extract against fungi *Aspergillus niger* and *Penicillium* sp. and to determine the effect of extract addition on the quality of wheat bread.

2. Materials and Methods

2.1. Fungal Pathogens

Pure cultures of *Aspergillus niger* and *Penicillium* sp., isolated from mouldy bread, were obtained from the Department of Analysis and Food Quality Assessment of the University of Life Sciences in Lublin, Poland. The cultures of fungi were kept fresh and viable by periodical transfers on YPD medium (10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar per 1 L of distilled water; pH 6.0 ± 0.2) under aseptic conditions throughout the study. Strains were stored at 4 °C for routine cultivation.

2.2. Plant Material

Dried petals of *Calendula officinalis* originating from organic farming (Dary Natury, Grodzisk, Poland) were purchased at a local pharmacy and stored at room temperature.

2.3. Preparation of Plant Water Extract

Calendula officinalis flower water extract was prepared using the method described by Gonelimali et al. [15], with some modifications. The extraction was done using deionised water with a sample-to-solvent ratio of 1:20 (*w/v*). The dried material was powdered in a laboratory mill (IKA A11 basic, IKA-Werke GmbH & Co. KG, Staufen, Germany) until it passed through a 0.35 mm sieve. Twenty g of powder was soaked in distilled water in a round bottom flask, heated for 30 min at 90 °C, and then incubated for 24 h at 37 °C and 150 rpm in a shaking incubator. The obtained extracts were centrifuged for 10 min at 10,000 rpm and filtered using Whatman No. 1 filter.

2.4. Qualitative Phytochemical Screening

The water extract of *Calendula officinalis* flowers was evaluated for qualitative determination of major phytochemicals, including alkaloids, saponins, tannins, flavonoids, glycosides, terpenoids, and reducing sugars according to the methodology described below [16–18]. Specific reagents were used for each chemical group to induce chemical reactions that developed distinct colours and/or precipitates characteristic of each class of substances.

Test for alkaloids: A few drops of sulfuric acid were added to the 3 mL of extract, and the formation of orange colour indicated the presence of flavonoids.

Test for saponins: 3 mL of extract was shaken vigorously with 3 mL of distilled water in a test tube, and the mixture was warmed. The formation of stable foam was taken as an indication of the presence of saponins.

Test for tannins: 3 mL of the extract was mixed with 3 mL of water and heated in a water bath. Then ferric chloride was added to the mixture. The dark green colour indicated the presence of tannins.

Test for flavonoids: A few drops of lead acetate solution were added to the 3 mL extract. A yellow-colour precipitate indicated the presence of flavonoids.

Test for glycosides: To 2 mL of the extract, 3 mL of glacial acetic acid, one drop of 5% FeCl_3 and concentrated H_2SO_4 were added. A reddish-brown colour appeared at the junction of the two liquid layers, and the top layer was blue-green, indicating the presence of glycosides.

Test for terpenoids: 3 mL of the extract was mixed with 2 mL of chloroform and 3 mL of concentrated sulfuric acid added carefully to form a layer. A reddish-brown colour indicated the presence of terpenoids.

Test for reducing sugars: To 0.5 mL of extract, 5 mL of Benedict's reagent was added. Then the mixture was boiled for 5 min. The presence of a bluish-green precipitate indicated the presence of reducing sugars.

2.5. Phenolic Content of *Calendula officinalis* Water Extract

The total phenolic content (TPC) of the extract was determined using Folin-Ciocalteu Reagent (FCR) as described by Singleton and Rossi [19]. To 0.1 mL of the extract were added 3 mL of distilled water and 0.4 mL FCR. After 3 min, 1.5 mL of sodium carbonate (100 g/L) was added, and the contents were mixed and allowed to stand for 30 min. The absorbance of the resulting solutions was measured at 760 nm using a UV-VIS spectrophotometer (Cary 50 Scan, Varian, USA) against a reagent blank. The concentration of TPC was calculated from a standard curve constructed using gallic acid solutions following the method described above. The range of gallic acid standard concentration was between 0 and 1 g/L. The TPC in the extract was expressed as gallic acid equivalents (GAE) in mg/g of dry weight (DW). The experiment was performed in triplicate.

2.6. Antifungal Activity of *Calendula officinalis* Water Extract In Vitro

2.6.1. Direct Contact Assay

The effect of the *Calendula officinalis* water extract on mycelial growth was studied according to the methodology described by Gleń and Boligłowa [20]. Twenty millilitres of YPD medium supplemented with *Calendula officinalis* water extract (5 mg/mL) at 5%, 10%, and 15% (v/v) were poured into sterilized Petri dishes (inner diameter 90 mm). After the medium had solidified, it was inoculated centrally with a 10-day-old culture of fungi *A. niger* and *Penicillium* sp. discs (5 mm in diameter). Control was the Petri dishes with pure YPD medium inoculated similarly. The Petri dishes were incubated at 28 °C. The experiment was performed in triplicate.

The effect of *Calendula officinalis* water extract on the mycelial growth of the tested fungi after 3, 5, and 7 days was calculated from the formula:

$$\text{Inhibitory activity \%} = (C - T)/C \times 100\%$$

where C and T are the average diameters (mm) of fungal mycelia in the control and the treatment, respectively.

2.6.2. Influence of *Calendula officinalis* Water Extract on Fungal Biomass Production Preparation of Spore Suspension of *Aspergillus niger* and *Penicillium* sp.

Aspergillus niger and *Penicillium* sp. were kept on YPD medium plates at 28 °C. Spores of fungus were harvested from 10-day-old cultures in Ringer's solution (peptone 1 g, NaCl 8 g per 1 L of distilled water; pH 6.9 ± 0.2) and loosened by 15 min shaking. The suspension was adjusted to 1 × 10⁶ spores/mL.

Fungal Biomass Production

The biomass growth of tested fungi was carried out in 200 mL Erlenmeyer flasks per 100 mL of YPG medium (10 g yeast extract, 20 g peptone, 20 g glucose per 1 L of distilled water; pH 6.0 ± 0.2), in which part (5%, 10%, and 15%) was replaced with a previously prepared *Calendula officinalis* water extract, according to the methodology of Gleń and Boligłowa [20] with some modifications. One mL of the prepared spore suspension of the tested fungi was added to each flask. The control was the flask with pure YPG medium inoculated in the same way. The flasks were incubated at 28 °C for 7 days and at 150 rpm in a shaking incubator. After the incubation period, the contents of each flask were filtered through standard filter paper (each filter had been weighed). The harvested mycelium was dried at 80 °C until a constant weight was obtained. The fungal biomass of each treatment was weighed and compared with the control. The experiment was performed in triplicate.

The influence of *Calendula officinalis* water extract on fungal biomass production was calculated by using the formula:

$$\text{Inhibitory activity \%} = ((W_{bc} - W_{bt})/W_{bc}) \times 100\%$$

where: W_{bc} is the weight of biomass control, and W_{bt} is the weight of biomass treatment.

2.7. Effect of the *Calendula officinalis* Water Extract Addition on Wheat Bread Quality

2.7.1. Laboratory Baking

The control bread was prepared from wheat flour type 750 (150 g), water (90 mL), compressed baker's yeast (4.5 g), saccharose (1.5 g), and salt (3 g). The study material was wheat bread made of dough, in which part of the water (10% and 15%) prescribed by the recipe was replaced with a previously prepared *Calendula officinalis* water extract (5 mg/mL). The ingredients were mixed, and the dough was formed for 50 min at 35 °C. Bread loaves were baked for 25 min at 210 °C, then immediately transferred to the laminar flow cabinet and cooled under sterile conditions. Bread loaves were weighted, transferred into plastic bags (one loaf per bag), sealed, and stored in room conditions (until the next day) for further analysis.

2.7.2. Bread Quality Characteristics

The prepared bread dough was weighed, and the dough yield (Y_d) was calculated according to the formula:

$$Y_d (\%) = (W_d \times 100\%) / F_w$$

where: W_d is the weight of dough (g), and F_w is the weight of used flour (g).

The cooled bread (24 h after baking) was weighed, and the bread yield (Y_b) was calculated according to the formula:

$$Y_b (\%) = (W_b \times Y_d) / W_d$$

where: W_b is the weight of bread after cooling (g), Y_d is the dough yield (%), and W_d is the weight of dough (g).

The total baking loss (Bl) was calculated according to the formula:

$$Bl (\%) = ((W_d - W_b)) / W_d \times 100\%$$

where: W_d is the weight of dough (g), and W_b is the weight of bread after cooling (g).

Bread loaf volume was measured 24 h after baking using the millet (*Cenchrus americanus* L.) seed displacement method. Specific volume (SV) was calculated as the loaf volume divided by weight. The Polish standard determined bread acidity and crumb moisture (PN-A-74108: 1996). To determine acidity, 25 g of bread was weighed into flasks each and 250 mL of distilled water at about 60 °C was added, then closed tightly with a rubber stopper. The sample was shaken for 3 min, then allowed to stand for another 3 min and shaken again for 1 min. The suspension was filtered through cotton wool, and 50 mL of filtrate was taken from each sample into a conical flask. It was titrated with 0.1 M NaOH solution in the presence of a few drops of 1% phenolphthalein until the pink color of the solution appeared and persisted for 30 s. The result was calculated according to the formula:

$$x_5 = 2a$$

where: x_5 —bread acidity [°], a —volume of 0.1 M NaOH solution used for titration [mL].

2.8. Phenolics Content and Antioxidant Potential of Bread

2.8.1. Extraction of Bioactive Compounds

The bread samples (1 g) were ground, placed in Falcon tubes, and shaken with 10 mL of a 4:1 (v/v) ethanol/water mixture for 120 min in a laboratory shaker. Then, the samples were centrifuged at 3000 rpm for 10 min. The supernatant was stored at −18 °C for further analysis.

2.8.2. Phenolics Content

The TPC of the bread extract was determined using FCR as described in Section 2.5. and the results are expressed as gallic acid equivalents in mg/g of bread.

2.8.3. DPPH (2-Diphenyl-1-Picrylhydrazyl) Radical Scavenging Activity

The DPPH assay was determined according to Shahidi et al. [21], with some modifications. A 0.15 mL of the prepared extract was mixed with 2.85 mL of a 0.1 mM solution of DPPH• in 75% methanol. The absorbance was measured after 30 min of reaction at 517 nm. The scavenging percentage was calculated using the formula:

$$\text{Scavenging activity \%} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100\%$$

where A_{sample} is the absorbance of the mixture of sample and DPPH•; A_{control} is the absorbance of the control (DPPH• solution).

2.8.4. ABTS (2,2'-Azobis(3-Etylobenzotiazolino-6-Sulfoniano)) Radical Scavenging Activity

The ABTS assay was determined according to Re et al. [22], with some modifications. The radical solution was prepared with ABTS and potassium persulfate, diluted in water to a final concentration of 2.45 mM, and left in the dark for 16 h to allow for radical development. The solution was diluted to reach the absorbance measures around 0.7 at 734 nm. Then, 2.85 mL of the ABTS•+ solution was mixed with 0.15 mL of each sample. The absorbance was measured after 30 min of the reaction at 734 nm. The scavenging percentage was calculated using the formula:

$$\text{Scavenging activity \%} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100\%$$

where A_{sample} is the absorbance of the sample and ABTS•+; A_{control} is the absorbance of the control (ABTS•+ solution).

2.9. Microbiological Quality of Bread

The number of yeasts and moulds per 1 g of bread was determined using the plate technique on YGC medium (5 g yeast extract, 20 g glucose, 0.1 g chloramphenicol, 15 g

agar per 1 L of distilled water; pH 6.6 ± 0.2). The assay was performed on the first day (after cooling) and after 7 days of storage in sterile Petri dishes at 28 °C. To isolate yeasts and moulds from bread, 10 g of crumbs were collected under sterile conditions under a laminar chamber into 300 mL conical flasks containing 90 mL of sterile Ringer's solution. The flasks were shaken at 180 rpm for 10 min. Then serial dilutions were made, and 1 mL was transferred to sterile Petri dishes containing YGC medium with chloramphenicol. The flasks were incubated for 5 days at 28 °C. After incubation, the colonies were counted and expressed as colony-forming units per gram (cfu/g) of samples. The test was performed in triplicate for each sample.

2.10. Consumer Evaluation

Consumer evaluation of bread was conducted on 29 consumers (18–25 years old). The bread was cut into slices about 1.5 cm thick the day after baking and coded with a three-digit number. The external appearance, crust (appearance and connection with the crumb; colour; thickness), crumb (appearance; colour; porosity; elasticity), smell, and taste were assessed. The bread was evaluated using the 5-point quality scale method. Grade 5 meant very good quality, grade 4—good quality, grade 3—satisfactory quality, grade 2—unsatisfactory quality, and grade 1—bad quality. The evaluation was based on a table with assigned definitions to each scale of scores for individual bread characteristics. Each quality discriminant has assigned an appropriate weighting factor (external appearance 0.1; crust 0.1; crumb 0.2; smell 0.2; taste 0.4). Plain water was used for mouth rinsing before and after each sample test.

2.11. Statistical Analysis

The results were analyzed statistically using Statistica 13.3 (StatSoft, Cracow, Poland) and Excel 2019 (Microsoft, Washington, DC, USA). A one-way analysis of variance (ANOVA) was carried out to compare the results, and the significance of differences between group means was determined using Tukey's post hoc test. All statistical hypotheses were verified at the significance level of $p < 0.05$.

3. Results and Discussion

3.1. Qualitative Phytochemical Screening

The results of qualitative phytochemical screening of the *Calendula officinalis* water extract are shown in Table 1. The preliminary phytochemical test results indicated the presence of alkaloids, saponins, flavonoids, glycosides and terpenoids in the tested extract.

Table 1. Phytochemical screening of the *Calendula officinalis* flower water extract.

Phytochemicals	Alkaloids	Saponins	Tannins	Flavonoids	Terpenoids	Glycosides	Reducing Sugars
Test result	+	+	—	+	+	+	—

“+” presence and “—” absence of phytochemicals in the *Calendula officinalis* water extract.

The results agree with the literature data [23,24] and indicate that marigold can be considered a potential raw material for the extraction of bioactive components. *Calendula officinalis* is an important medicinal plant with diverse phytochemicals and biological activities, such as antioxidant, anti-inflammatory, and antimicrobial. Among the phytochemicals in marigold petals, triterpenes, flavonoids, phenolic acids, quinones and coumarins, and carotenoids are reported to play the most important role [25]. These compounds are responsible for antimicrobial activity, and their mechanisms of action are diverse, including inhibition of nucleic acid synthesis, interference with cytoplasmic membrane function, and slowing of microbial metabolism [26].

3.2. Total Phenolic Content of *Calendula officinalis* Water Extract

The TPC determined in the *Calendula officinalis* water extract was 12.8 ± 0.007 mg GAE/g DM.

Using various solvents and extraction conditions, many researchers have attempted to extract phenolic compounds from *Calendula officinalis*. Sytar et al. [27] showed that among the tested methanolic extracts of leaves of the representative family *Asteraceae*, marigold leaf extracts have been shown to have the highest total content of these compounds (1.125 ± 0.153 mg/g DM). A study by Olennikov and Kashchenko [28] reported that the TPC in the ethanolic extract of marigold leaves ranged from 29.21 to 50.24 mg/g, depending on the variety. In a study by Piekut [29], the ethanolic extract of marigold flowers had a low TPC content of 6.19 mg/g DM. Petkova et al. [30] found that calendula infusion showed a TPC of 16.49 ± 0.20 mg/g DM, while the decoction had a higher content of these compounds (19.44 ± 0.04 mg/g DM). Water extracts of *Calendula officinalis* flowers and leaves were studied by Mubashar Sabir et al. [31], showing that the TPC was 72.91 ± 2.1 mg/g and 23.1 ± 0.8 mg/g, respectively. Krochmal-Marczak and Kiełtyka-Dadasiewicz [32] found a significantly lower polyphenols content ranging from 0.06 to 3.64 mg/100 g DW in an infusion of marigold flowers, depending on brewing time and temperature.

Comparing the obtained results with the above reports by other authors, it can be concluded that the TPC in the plant extract is affected by many factors. These include the type of solvents used, their ratio, extraction time and temperature, the chemical composition and physical properties of the plant materials, and even the part of the plant used (flowers, leaves). Data available in the literature state that the chemical compounds found in plants are also influenced by the region where the plant is grown, climatic conditions (e.g., rainy and dry seasons), growing season (beginning or end of flowering), and genetic factors [27,33]. Therefore, all these factors should be considered when comparing the TPC in an extract from a given plant. The repeatability of chemical composition is one of the main problems in using plant extracts as food preservative ingredients. Extracts of natural origin, or different batches of extracts produced by different manufacturers, can exhibit quantitative and qualitative variability.

3.3. Antifungal Activity of *Calendula officinalis* Water Extract In Vitro

3.3.1. Direct Contact Assay

The study found that adding *Calendula officinalis* water extract to the medium limited the mycelial growth of *A. niger* after 3, 5, and 7 days of incubation on Petri dishes (Figure 1). The greatest inhibition was found in a Petri dish with the addition of 15% (v/v) of water extract in the medium, and it ranged from 49.81% on the 7th day of cultivation to 62.98% on the 3rd day. On the other hand, the tested extract did not affect inhibiting the mycelial growth of *Penicillium* sp. (Figure 2).

Numerous literature reports indicate the effectiveness of extracts prepared from various plant species, including marigolds, against pathogenic microorganisms [6,12,27,34,35]. Efstratiou et al. [12] found that methanolic and ethanolic extracts of *Calendula officinalis* petals showed excellent antifungal activity against 9 different species from genera *Candida*, *Aspergillus*, and *Exophiala*. Similar to the present study, the researchers proved that marigold extract reduced the growth of *A. niger* on Petri dishes [12]. Rigane et al. [36] also showed such activity for water-methanol extracts from calendula flowers and leaves. Ikeura et al. [37] screened the antifungal activity of extracts from 16 plants (dichloromethane and diethyl ether were the solvents) against *Penicillium expansum* and showed that marigold extract strongly reduced the growth of the pathogen by the steam contact method.

The antimicrobial properties of *Calendula officinalis* are due to the presence of numerous bioactive compounds; among them, phenolic compounds play an important role in the control of fungi [38]. The literature shows that their antimicrobial activity is based on different mechanisms due to the wide variation in the structure of these substances. Phenolic compounds can interact with the cytoplasmic membrane, cell wall, nucleic acids,

and energy transport, altering or inhibiting their functions. They also denature enzymes or bind vitamins, minerals, or carbohydrates, making these compounds unavailable to microorganisms [39].

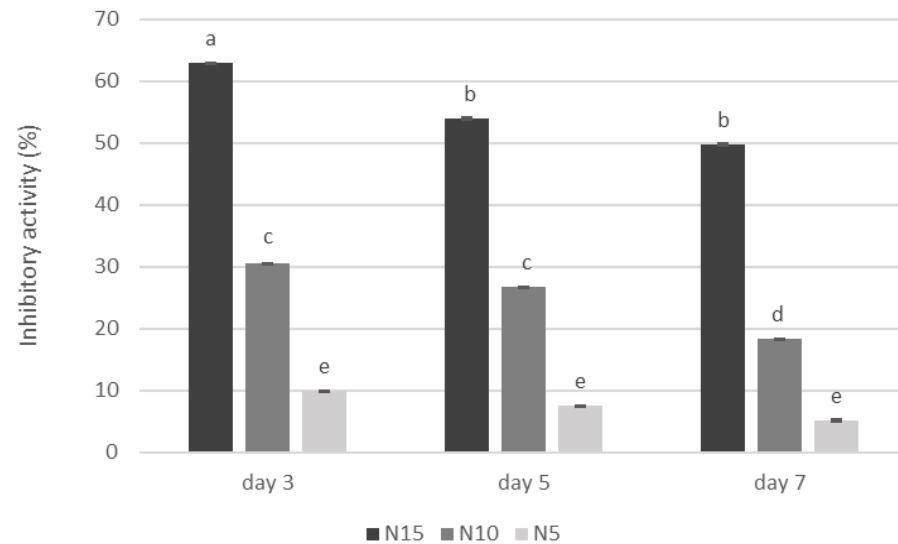


Figure 1. Effect of adding *Calendula officinalis* water extract on the mycelial growth of *Aspergillus niger*. N15, N10, N5—addition of *Calendula officinalis* water extract to the medium in doses of 15%, 10%, and 5% (v/v), respectively; Values marked with the same letters do not differ significantly at $p < 0.05$ (Tukey's post hoc test); mean value ($n = 3$) $\pm$ SD.

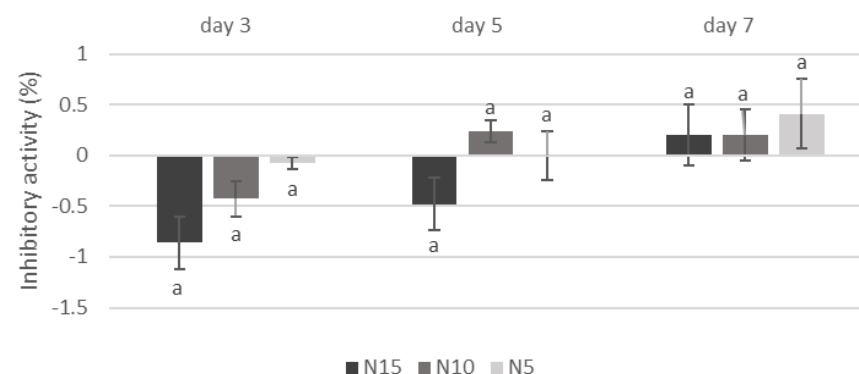


Figure 2. Effect of adding *Calendula officinalis* water extract on the mycelial growth of *Penicillium sp.* N15, N10, N5—addition of *Calendula officinalis* water extract to the medium in doses of 15%, 10%, and 5% (v/v), respectively; Negative values denote stimulation of fungal biomass production; Values marked with the same letters do not differ significantly at $p < 0.05$ (Tukey's post hoc test); mean value ($n = 3$) $\pm$ SD.

3.3.2. Influence of *Calendula officinalis* Water Extract on Fungal Biomass Production

There was a significant inhibition of *A. niger* biomass production after 7 days of incubation when 15% and 10% *Calendula officinalis* water extract were added to the culture medium. The reduction in pathogen biomass was 15.50% and 9.17%, respectively. On the other hand, as in the previous experiment on Petri dishes, the study extract did not significantly affect the inhibition of *Penicillium sp.* biomass production.

Studies on the inhibitory effect of plant extract on the growth of pathogenic fungi biomass have been reported by some researchers. Bajwa et al. [40] evaluated the potential of water extracts of *Parthenium hysterophorus*, a plant in the *Asteraceae* family, against three pathogenic fungi: *Drechslera tetramera*, *Phoma glomerata*, and *A. niger*. The lower extract concentrations proposed in the experiment caused a decrease in fungal biomass production,

and this response was species-specific. In contrast, increasing extract concentrations increased fungal biomass production at all harvest intervals. Gleń and Boligłowa [20] showed that water extracts of walnut (*Juglans regia*) leaves, birch (*Betula verrucosa* Ehrh.) bark, and nettle (*Urtica dioica* L.) herb strongly reduced the biomass production of *Fusarium culmorum* (29.60–53.70%) and *Botrytis cinerea* (17.90–53.80%), while in some variants of the experiment tested extracts showed a stimulating effect on the biomass growth of *Alternaria alternata*, *Phoma exigua*, and *Sclerotinia sclerotiorum*. El-Mohamedy and Abdallah [41] tested the anti-fungal activity of *Moringa oleifera* Lam. seed oil and extract against *Fusarium oxysporum*, *F. solani*, *Alternaria solani*, *A. alternata*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina*, and the researchers showed that study oil and extract had an inhibitory effect on the growth of pathogen biomass.

The results presented in the paper and reports by other researchers indicate that a particular plant extract can reduce the biomass production of one pathogen and have no effect or even stimulate the growth of another. Therefore, testing a broad spectrum of product-specific microorganisms is appropriate when designing new foods with plant extracts. This will make it possible to create foods of high quality and high safety for the consumer.

3.4. Bread Quality Characteristics

Due to the low fungistatic activity obtained with the 5% addition of *Calendula officinalis* water extract in the culture medium, it was decided to use only the higher addition in bread production and replace the water recommended by the recipe with 10% and 15% extract. The evaluation of the control and bread with the addition of extract quality parameters is shown in Table 2. The appearance of the prepared bread is shown in Figure 3.

Table 2. Evaluation of bread quality parameters.

Parameters	Sample		
	Control	N10	N15
Bread yield (%)	139.54 ± 2.78 ^a	139.42 ± 2.60 ^a	140.49 ± 0.73 ^a
Baking loss (%)	11.14 ± 1.11 ^a	10.68 ± 0.39 ^b	9.16 ± 1.46 ^c
Specific volume (cm ³ /g)	2.12 ± 0.19 ^b	2.67 ± 0.17 ^a	2.61 ± 0.23 ^a
Crumb moisture (%)	31.64 ± 3.29 ^b	37.26 ± 5.40 ^a	37.49 ± 5.89 ^a
Acidity [°]	1.73 ± 0.12 ^c	2.00 ± 0.00 ^b	2.23 ± 0.03 ^a

N10, N15—bread, in which 10% and 15% of the water have been replaced with *Calendula officinalis* water extract, respectively. Within each row, values with the same letter do not differ significantly at $p < 0.05$ (Tukey's post hoc test); mean value ($n = 3$) ± SD.

The addition of *Calendula officinalis* water extract used in the study did not affect bread yields, and the results were similar to the control. The experiments found that bread with the extract showed significantly lower baking losses compared to the control. The total baking loss depends on numerous factors, including the size, shape, volume, and type of bread, dough management and baking method, and even the type of oven used [42]. Alotaibi et al. [43] report that the lower baking losses in bread with the addition of marigold may be due to the presence of a large amount of lutein, which binds to water and thus reduces moisture loss. Accordingly, it was found that the enriched bread had a significantly higher (about 18%) moisture content compared to the control. The bread with extract also had significantly higher volume than the control, by about 23% and 26% in the bread, in which 15% and 10% of the water was replaced with *Calendula officinalis* extract, respectively. The specific bread volume is considered one of the main characteristics defining consumer acceptability [43], so the results obtained in this paper are significant. In contrast to the present study, Alotaibi et al. [43] found that enriching bread with dried marigold flower

powder reduced volume. In the experiments presented here, it was also noted that the acidity of the bread increased as the addition of extract increased.

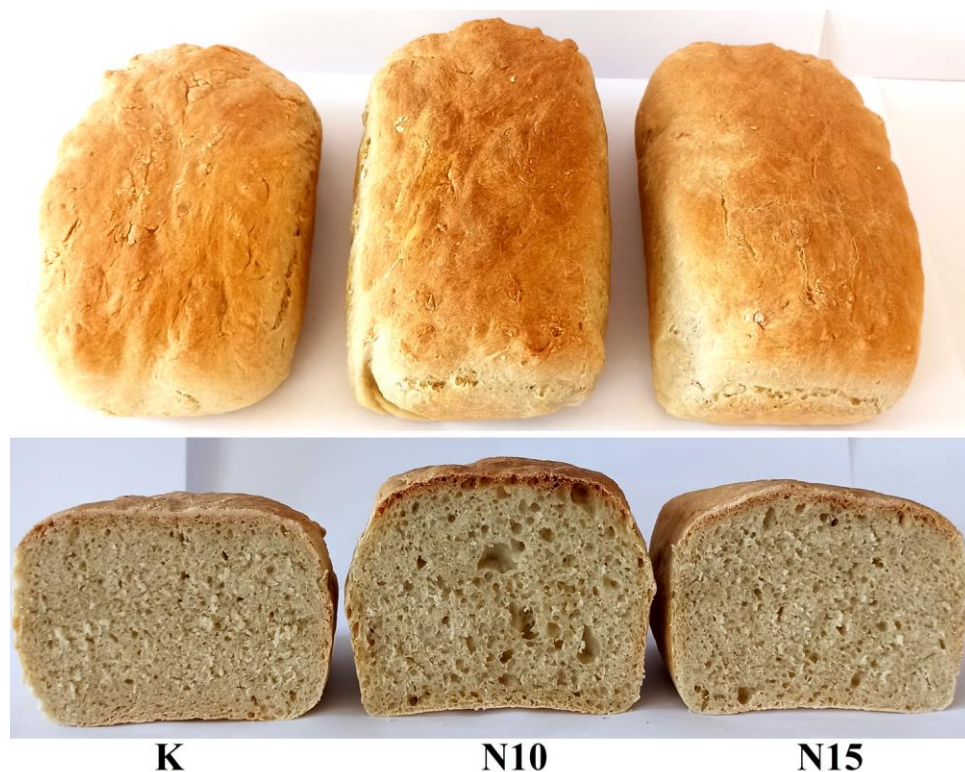


Figure 3. The appearance of control bread (K) and with the addition of *Calendula officinalis* water extract, 10% (N10) and 15% (N15), respectively.

Dough expansion depends on dough rheological characteristics, primarily due to the gluten and starch components. Adding bioactive ingredients may physically modify the development of bread dough [44] since antioxidants can form complexes with proteins and/or polysaccharides. Several studies have shown an adverse effect of the powdered plant material addition on bread volume [45]. This can be explained by the presence of fibre, which interacts with proteins and weakens the gluten network of the dough, resulting in a reduction in the amount of CO₂ retained in the dough during fermentation [46]. Jakubczyk et al. [47] report that *Calendula officinalis* has a high total fibre content (62.33 ± 9.17 g/100 g), but most of the fraction is insoluble (57.54 ± 8.32 g/100 g). In general, insoluble fibres reduce the technological quality of baked goods, while soluble fibres have a positive effect [48], so using water extract was reasonable in the present study.

3.5. Phenolics Content and Antioxidant Potential of Bread

The effect of adding *Calendula officinalis* water extract on the TPC and antioxidant activity of wheat bread is presented in Table 3. As expected, marigold extract positively affected fortified bread's phenolic content and antioxidant status. Compared to the control, the TPC was significantly higher by about 77% and 95% in the bread, in which 10% and 15% of the water was replaced with *Calendula officinalis* extract, respectively. In addition, the experiment observed significantly higher free radical scavenging activity for ABTS•+ than DPPH•, which may be related to their solubility in water and organic solvents. The same relation was found in other studies for marigold flower extract [23,30]. In the present study, the antioxidant potential against DPPH• was significantly higher in both enriched bread than bread without extract. In this case, the level of antioxidant activity increased with the addition of marigold water extract. In contrast, the antiradical activity against

ABTS•+ was higher only in bread, in which 15% of the water was replaced by *Calendula officinalis* extract.

Table 3. Phenolics content and antioxidant activity of wheat bread with *Calendula officinalis* water extract.

	Sample		
	Control	N10	N15
TPC (mg GAE/g DW)	0.44 ± 0.00 ^b	0.78 ± 0.01 ^a	0.86 ± 0.01 ^a
DPPH (%)	6.87 ± 0.01 ^c	8.47 ± 0.01 ^b	10.40 ± 0.01 ^a
ABTS (%)	20.24 ± 0.01 ^b	20.53 ± 0.01 ^b	33.59 ± 0.02 ^a

N10, N15—bread, in which 10% and 15% of the water have been replaced with *Calendula officinalis* water extract, respectively. TPC—total phenolic contents; ABTS—the ability to quench ABTS radicals; DPPH—the ability to quench DPPH radicals; Within each row and for the selected assays, values with the same letter do not differ significantly at $p < 0.05$ (Tukey's post hoc test); mean value ($n = 3$) ± SD.

Food fortification is of increasing interest to consumers. Many products are enriched to improve their phenolic levels and antioxidant potential, thereby increasing their health-promoting properties. This is particularly important for bread derived from white wheat flour. Edible flowers are a good source of compounds with antioxidant activity [10], and *Calendula officinalis* has been extensively studied in this aspect [23,27,30]. Measuring the antioxidant capacity can provide a wealth of information on the oxidation resistance, the quantitative contribution of antioxidants, or the antioxidant effects that may occur in the body during consumption [49]. The radical-scavenging properties of natural compounds are often associated with their ability to quench free radicals. Many literature reports confirm that adding plant extracts increases bread's polyphenol content and antioxidant activity [50,51], and our results indicate the potential of *Calendula officinalis* in food fortification.

3.6. Microbiological Quality of Bread

After 7 days of bread storage in Petri dishes at 28 °C, there was no visual evidence of microbial spoilage. However, after the isolation of yeasts and moulds from the bread, their presence was found in all samples, but their amount was within acceptable standards for bread (Table 4). The highest number of yeast and mould was found in the control, both on the baking day (1.50×10^2 cfu/g) and after 7 days of storage (2.30×10^2 cfu/g). Similar numbers of these microorganisms (2.20×10^2 cfu/g) were found after storage in bread with 15% of the prepared extract. Conversely, the lowest number of yeast and mould on a baking day (1.23×10^2 cfu/g) as well as after 7 days of storage (1.90×10^2 cfu/g) was observed in bread in which 10% of the water was replaced with the *Calendula officinalis* extract.

Table 4. Number of yeasts and moulds in bread.

Sample	Number of Yeasts and Moulds in Bread (cfu/g)	
	Baking Day	After 7 Days of Storage
Control	$1.50 \times 10^2 \pm 0.05^a$	$2.30 \times 10^2 \pm 0.04^a$
N10	$1.23 \times 10^2 \pm 0.06^b$	$1.90 \times 10^2 \pm 0.06^b$
N15	$1.30 \times 10^2 \pm 0.03^b$	$2.20 \times 10^2 \pm 0.05^a$

N10, N15—bread, in which 10% and 15% of the water have been replaced with *Calendula officinalis* water extract, respectively; Within each column and for the selected day, values with the same letter do not differ significantly at $p < 0.05$ (Tukey's post hoc test); mean value ($n = 3$) ± SD.

One of the difficult challenges facing the bakery industry is to reduce microbial spoilage, especially fungi, thereby extending shelf life and guaranteeing product safety. It has been proven that some plant extracts can inhibit the growth of pathogenic microor-

ganisms, so enriching bread is reasonable. Pinilla et al. [52] found that bread fortified with garlic extract was more microbiologically stable compared to the control, and the addition of the extract inhibited the growth of the fungi *A. flavus*, *Penicillium herquei*, and *F. graminearum* in the bread. A study by Douadi et al. [53] proved that cinnamon (*Cinnamomum verum*) extract improves the shelf life of bread, delays spoilage, and reduces the growth of *Aspergillus* sp. fungi. Numerous works by other researchers have prompted the search for natural bread additives to extend the shelf life of the finished product. Several reports in the literature about the effectiveness of marigold extract against fungi of the *Aspergillus* and *Penicillium* genera [12,36,37] pose a threat in baking. The results presented in this paper prove that the appropriate addition of *Calendula officinalis* water extract can be successfully applied to bread and positively affect the microbiological quality of the product.

3.7. Consumer Evaluation

The average consumer ratings of the bread from the individual parameters were similar in each sample and were not statistically significantly different from each other (Table 5). The control and enriched bread received similar overall ratings and were of good quality, according to consumers. The control bread received an overall score of 3.96, the bread in which 10% of the water was replaced with prepared *Calendula officinalis* water extract received 4.02, and in the case of 15% replacement of water with extract 3.94. The results show that the addition of *Calendula officinalis* water extract used in the experiment had no significant effect on the organoleptic properties of the bread.

Table 5. Sensory evaluation of bread prepared with *Calendula officinalis* water extract.

Evaluation Parameters		Control	N10	N15
External appearance		4.14 ± 0.64 ^a	4.10 ± 0.72 ^a	4.00 ± 0.76 ^a
Crust	Appearance and connection with the crumb	4.10 ± 0.62 ^a	4.09 ± 0.70 ^a	4.00 ± 0.89 ^a
	Color	4.10 ± 0.77 ^a	4.07 ± 0.88 ^a	3.86 ± 0.95 ^a
	Thickness	4.14 ± 0.74 ^a	3.93 ± 0.88 ^a	4.14 ± 0.79 ^a
Crumb	Appearance	4.07 ± 0.70 ^a	4.00 ± 0.80 ^a	4.17 ± 0.80 ^a
	Color	3.93 ± 0.80 ^a	4.14 ± 0.83 ^a	4.14 ± 0.95 ^a
	Porosity	3.97 ± 0.73 ^a	3.83 ± 1.04 ^a	4.14 ± 0.83 ^a
	Elasticity	3.83 ± 0.85 ^a	3.86 ± 0.83 ^a	3.86 ± 0.89 ^a
Aroma		4.07 ± 0.84 ^a	4.07 ± 0.80 ^a	4.07 ± 0.88 ^a
Taste		3.83 ± 0.71 ^a	4.00 ± 0.85 ^a	3.79 ± 0.90 ^a
Overall		3.96 ± 0.12 ^a	4.02 ± 0.10 ^a	3.94 ± 0.14 ^a

N10, N15—bread, in which 10% and 15% of the water have been replaced with *Calendula officinalis* water extract, respectively; Within each row and for each factor, values with the same letter do not differ significantly at $p < 0.05$ (Tukey's post hoc test); mean value ($n = 29$) ± SD.

The attractiveness of bread consists of many different characteristics, but the first to attract the consumer's attention is external appearance and colour. Edible flowers or extracts from them can be an interesting alternative to artificial colours used in food technology [54]. The obtained *Calendula officinalis* extract was brown, but the amount of additive used was low enough that it did not significantly change the colour of the finished product. The addition of plant extracts rich in phenolic compounds, which aim to increase the antioxidant capacity of wheat bread, can also affect certain rheological properties of the dough, such as gumminess, strength, elasticity, adhesiveness, and chewiness, among others [1]. As a result, the sensory properties of the final product are also modified, which is not always acceptable to consumers. For example, in a study by Czaja et al. [51], according

to consumers, bread enriched with onion extract had a less acceptable smell and taste than the control bread. In the development of functional foods, a key role is played by finding ingredients that allow specific beneficial effects to be achieved without modifying the sensory properties of the enriched product. Therefore, the results obtained in the present study are significant and encourage further research into using *Calendula officinalis* water extract for bread fortification.

4. Conclusions

The demand for natural, chemical-free, “clean label” products induces the search for new methods to extend food shelf life and eliminate harmful microorganisms from food products. Plants extracts are an active area for research due to their rich phytochemical composition. *Calendula officinalis* is widely used in cosmetics, while the present study demonstrated the potential of this plant in food fortification. The studied marigold extract showed antifungal properties against *A. niger*, and the enriched bread had satisfactory quality characteristics, exhibited antiradical activity, and had unchanged sensory properties. The results encourage further research into using *Calendula officinalis* in food technology.

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