Research article

Influence of *Tagetes patula* and *Viola tricolor* on survival of *Staphylococcus aureus* ATCC 25923

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**HIGHLIGHTS**
- The reduction in staphylococci number by macerates of the petals and mixtures didn’t exceed 11% of the inoculum value.
- The results obtained by the disc diffusion method showed a weak biostatic effect of both individual flowers and mixtures.

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**ABSTRACT**

So far, no studies have assessed the antibacterial properties of macerates of flower petals intended for human consumption. Previous studies have focused on the role of extracted flower components in inhibiting bacterial growth, not considering the petal tissue as a mixture of different components. The aim of this study was to assess the inhibitory effect of unpreserved macerates and juices derived from edible flower petals of *Viola tricolor* and *T. patula* on the population of *Staphylococcus aureus* ATCC 25923. Evaluation of the biostatic properties of flowers was carried out in two stages: using the Baird-Parker RPF culture method and the disc diffusion method. The reduction in the number of staphylococci by the macerates of the petals and their mixtures did not exceed 11% of the inoculum value. A low degree of inhibition of *S. aureus* ATCC 25923 was found with *T. patula* macerate and sap in studies using both methods. The disc diffusion method in the study revealed the synergistic effect of the petals of both species on *S. aureus* ATCC25923 cells.

1. Introduction

The consumption of edible flowers is a contemporary dietary trend (Benvenuti and Mazzonchini, 2021; Kumari et al., 2021). Studies on this subject are focused on their health-promoting properties, indicating the anti-inflammatory, anti-cancer, neuroprotective and anti-aging properties of edible flowers (Chitrakar et al., 2019; Kalemba-Drozd and Cierńszczyk, 2013; Kalemba-Drozd and Cierńszczyk, 2016; Kumari et al., 2021). Recently, the toxicological aspects related to the consumption of flowers have also been investigated (Drava et al., 2020; Kalemba-Drozd, 2019; Lu et al., 2016; Guiné et al., 2021). Some studies have focused on consumer acceptance and preferences related to the organoleptic characteristics of flowers as well as consumer knowledge about the preservation of flowers (Guiné et al., 2021; Simoni et al., 2018). However, data regarding the changes in the microbiological quality of flowers during postharvest storage are scarce, raising the question of the safety of these raw materials (Wilczyńska et al., 2021; Marchioni et al., 2020). The flowers of rose, bitter orange, violets, nasturtiums, pansies, and marigolds have been known and used in many regional cuisines for many years (Benvenuti et al., 2016; Kaleba-Drozd and Cierńszczyk, 2013; Kalemba-Drozd, 2016; Kumari et al., 2021; Pires et al., 2018).

Among the many types of known edible flowers, pansies (*Viola tricolor*), marigolds (*Tagetes patula*) and roses (*Rosa* spp.) are listed as the most common ingredients in dishes (Kaleba-Drozd and Cierńszczyk, 2013). The health-promoting or healing properties of flowers of the genera *Viola* spp. or *Tagetes* spp. are known and cited extensively in the literature, with emphasis on the antioxidant, immunosuppressive, neuroprotective and antimicrobial properties of selected ingredients (Faizi et al., 2008; Fernandes et al., 2019; Gautam and Kumar, 2017; Jain et al., 2012; Jha et al., 2012; Khoshkam et al., 2016; Latifian et al., 2021; Moliner et al., 2018; Prabawati et al., 2021; Youssuf et al., 2021).

However, there are no studies on the antibacterial properties of macerates of flower petals intended for direct consumption. Available data are mainly related to the role of the extracted flower components in inhibiting bacterial growth, not considering the petal tissue as a mixture...
of different components. In this context, the aim of this study was to evaluate the inhibitory effect of unpreserved macerates and sap derived from edible flower petals of *V. tricolor* and *T. patula* on *Staphylococcus aureus* ATCC 25923 populations.

2. Materials and methods

2.1. Plant material

We used flowers of the species *V. tricolor* and *T. patula* from the “Lawenda” (Horticultural Farm, Gdansk, Poland). In Lawenda, you can find over 200 different products - these are vast varieties of edible herbs and flowers. The flowers were harvested in June 2021, and the study was performed in four series in the laboratory of the Gdynia Maritime University.

2.2. Bacterial strain and culture conditions

The bacterial strain *S. aureus* ATCC 25923 was obtained from the Merck Collection (Poland), and a broth culture of *S. aureus* ATCC 25923 was prepared. The bacterial culture was obtained by reviving the lyophilisates in the liquid nutrient broth after 24–48 h of incubation at 37 °C. The inoculum was prepared on Baird-Parker RPF medium (bioMerieux, Poland). The obtained broth culture was poured into 8- and 5-mL test tubes.

2.3. Preparation of plant material and determination of microbial count

To determine the *Staphylococcus* count, 2 and 5 g of flowers were washed three times in sterile distilled water by transferring them from one container to the next. Subsequently, the flower petals were homogenised with a Stomacher Lab-Blender 400 (Seward, Worthing, UK) homogeniser to prepare the macerates. Biostatic activity was tested by the disc-diffusion method, and the petals were pressurised. Sap was pressed from 10 ± 1 g of flower petals. Flower petals adhered to the discs and were covered with aluminium foil; the layers prepared in this way were subjected to a pressure of 156 kPa (Zaidan et al. (2005) modified by Steinka and Voelkner). Flower macerates were added to broth cultures of *S. aureus* ATCC 25923, with initial population of 5.18–6.64 log CFU/mL.

2.4. Assessment of biostatic activity

Evaluation of the biostatic properties of flowers was carried out in two stages:

- Stage I - evaluation of the reduction of the number of *S. aureus* by the culture method in Baird-Parker RPF medium.
- Stage II - assessment of growth inhibition by the disc-diffusion method.

2.4.1. Culture-based method

The macerated flower petals, we transferred 2 g into 8 mL of *S. aureus* broth and 5 g into 5 mL of broth. After a reaction period of 30 and 120 min, 1 mL of the bacterial culture was collected, diluted and spread on Baird-Parker RPF medium, followed by incubation for 48 h at 37 °C. After incubation, the number of *S. aureus* ATCC25923 was determined in accordance with the methodology contained in PN-EN ISO 6888-1, 2001. The R staphylococcal number reduction index was determined to assess the reduction in the number of bacteria, using the following equation:

\[ R = K - \frac{N_{30\text{-}120}}{N_{0\text{-}120}} \times 100\% \]

where

- \( K \) - initial population of *S. aureus* ATCC25923,
- \( N_{30\text{-}120} \) - number of *S. aureus* ATCC25923 after 30 min of incubation,
- \( N_{120} \) - number of *S. aureus* ATCC25923 after 120 min of incubation.

2.4.2. Disc diffusion method

The antibacterial properties against *S. aureus* ATCC2523 (Merck, Poland) were determined by the disc diffusion technique. This method is based on the principle that the antimicrobial components of petals in the discs will diffuse into the media and inhibit the growth of sensitive organisms, thereby creating a zone around the disc. For this, 1 mL of *S. aureus* ATCC25923 culture with a known inoculum density was spread on a plate containing Agar Baird-Parker RPF ( bioMerieux, Poland). A suspension was prepared from the 24-hour bacterial culture; inoculum density was 5.97 log CFU/mL. The petals of each species of flowers and their mixtures were subjected to pressure, soaking their juice in paper discs with a diameter of 10 mm.

The discs were soaked with the juice of marigold (A), pansy (B), and a mixture of AB/BA (C) and BA/AB (D), where AB/BA were present in as follows (from the outer to the inner part): aluminium foil-marigold/pansy-disc-pansy/marigold-aluminium foil. For BA/AB, the order was aluminium foil-pansy/marigold-disc-marigold/pansy-aluminium foil (Figures 1 and 2).

After drying the plate with the bacteria, discs with a diameter of 10 mm, soaked in the juice of the petals, were placed onto the plate. Subsequently, using sterile tweezers, discs were subjected to natural juice of marigolds and pansies and a mix of both; pure discs were used as control. Each disc was gently pressed, ensuring even contact with the medium. Plates were incubated under aerobic conditions for 24–48 h at 37 °C. After incubation, a calliper gauge was used to determine the width of the microbial growth inhibition zone. Results are given as diameter of the zone growth inhibition in mm (Das-gupta et al., 2012). The degree of the sensitivity of staphylococci to the action of flowers was determined based on the size of the inhibition zone (CLSI, 2011).

2.5. Statistical analysis

We used the StataStatistical Software Release 15. StataCorp 2017 and Microsoft Excel – Microsoft Office Professional Office – Excel 2010 for data analysis. The results of four series of tests obtained in the first stage of the experiment were analysed using the paired t-test (mean comparison test) to determine whether the mean of a dependent variable is the same in the related groups. The results are expressed as mean and standard deviation. Correlation functions were calculated using the Statware office Excel 2010 software. Correlation equations were determined between the number of *S. aureus* during interaction with flower macerates and the time of co-incubation of bacteria with petals. The coefficients of determination of the equations illustrating the changes in the number of staphylococci, depending on the reaction time and the concentration of flower macerates, were determined.
3. Results and discussion

Evaluation of the number of S. aureus after interaction with Viola and Tagetes macerates and their mixtures showed that staphylococcal reduction and staphylococcal inhibition depended on the reaction time (Table 1). After 30 min of incubation, staphylococcal reduction by Tagetes was low (3.1%). The highest degree of reduction was observed at this time after the interaction of the bacteria with a 5% mixture of both flowers (V. tricolor and T. patula). Both time and concentration determined the behaviour of S. aureus during exposure to Viola macerate. A statistically significant difference was found for the 120 min incubation time of staphylococci with 2% Viola macerate, (mean = 1.23; SD = 0.57; P = 0.023). The concentration of the Viola macerate used in the experiment was statistically significant, with P = 0.008 after 120 min of incubation. For Viola, 97.6% of the observed reduction in the number of S. aureus was determined by the incubation time (\( R^2 = 0.976 \)) (Table 2). We observed no statistically significant difference for the effect of both T. patula concentrations on the number of S. aureus (\( P_{(T > 12)} = 0.392; P_{(T > 5)} = 0.485 \)). Similarly, no significant difference was found regarding the effect of interaction time on staphylococcal count reduction (\( P_{(T > 120)} = 0.16; P_{(T > 120)} = 0.04 \)).

The variability of the number of staphylococci under the influence of flowers could be described by a linear equation (Table 2). A 2.5-fold reduction in population size after interaction with the macerates was observed for the 5% flower mixture compared to the 2% mixture. Based on our results, factors other than time influenced the bacterial behaviour; only 15% of the change in number was determined by the total duration of the interaction between the macerate and the bacteria.

After 30 and 120 min of contact between the staphylococci and the macerates, we observed interactions between the components of both flowers. These interactions did not result in any further inhibition of the bacteria at the end of the experiment. The adjustment of the obtained data to the real conditions is illustrated by the second-degree polynomial equation (Table 2).

There is no information in the literature on the reduction in the number of S. aureus under the influence of macerates of edible flowers. The obtained data can be roughly compared with the results obtained for cold-water solutions of mixtures containing flowers. Evaluation of the biostatic properties of the aqueous solution of the mixture of holly and rose petals in interaction with S. aureus ATCC 25923 showed that the reduction in the number of staphylococci after 120 min was 8.2%. The observed decrease in the number of S. aureus was, on average, 0.5 log CFU/mL (Steinka, 2012, 2020). In the case of the multicomponent mixture of hibiscus and rosehip petals, the reduction was only 5.9% with a 0.5 McFarland inoculum. However, this mixture additionally contained cinnamon, cloves, dried apples, cherries and rosehips and orange peel in undefined proportions, suggesting that the components of the mixture could stimulate rather than inhibit the growth of staphylococcal cells. On the other hand, a high degree of inhibition of the S. aureus population was obtained when using an aqueous solution of Camelia sinensis with the addition of comflower and bergamot oil, reaching a reduction of 85.9%.

The recorded decrease in the number of staphylococci was probably the result of the synergy of the antibacterial components of tea as well as the oil fraction of bergamot and comflower flowers (Steinka, 2012, 2020). To date, the biostatic properties of edible flower macerates have not been investigated with the use of culture methods.

Our research shows that in the first phase of the interaction (30 min), the number of staphylococci decreased on average by 3.1–8.9% for both species of flowers, followed by 20% in the subsequent phase for V. tricolor. Instead of inhibition, a slight increase in the number of bacteria was also observed with the use of a higher concentration of Viola macerates, indicating the presence of Viola petal components in the broth medium during prolonged interaction (Table 1).

Based on the results of the disc diffusion method, there was a weak and moderate biostatic effect of both species and their mixtures (Table 3). The juice obtained during pressure maceration of the petals caused an inhibition zone with an average diameter of 8 mm for Viola and 14 mm for Tagetes. The disks were soaked with the mixture in two variants, when Viola (C) (Figure 2) or Tagetes (D) adhered to the surface of the disks during pressing. The observed changes in inhibition zones indicate a more effective biostatic effect of Viola juice directly adjacent to the disc (13.1 mm) compared to that observed for variant D (Table 3).

The zone diameters obtained by us were smaller compared to those obtained for the extracts of other edible flowers. For example, Evidente et al. (2004) assessed the effect of amaryllis extracts and obtained inhibition zones from 17 to 22 mm, indicating a high sensitivity of S. aureus.

It should be noted that these differences may have resulted from the use of substances extracted from flower petals and not their macerates. In this study, direct penetration of the surface of staphylococcal cells by the juice soaked in the disc was obtained. Also, S. aureus ATCC 25923 showed low sensitivity to the action of Viola, Tagetes and mixtures of both flowers (Table 3).

### Table 1. Changes in the number of S. aureus ATCC 25923 during interaction with flower macerates and their mixture [log CFU/g].

| Kind of samples | Macerate concentration [%] | Mean Standard Deviations | R30 [%] | Mean Standard Deviations | R120 [%] |
|-----------------|---------------------------|-------------------------|---------|-------------------------|---------|
| Tagetes patula  | 2                         | 5.44 ± 0.78             | 8.9     | 5.33 ± 0.70             | 10.8    |
| Tagetes patula  | 5                         | 5.19 ± 0.77             | 3.1     | 5.16 ± 0.80             | 3.6     |
| Viola tricolor  | 2                         | 5.52 ± 0.40             | 7.6     | 4.74 ± 0.63             | 20.7**  |
| Viola tricolor  | 5                         | 5.49 ± 0.62             | 8.1     | 6.13 ± 0.35             | 7.9**   |
| Tagetes patula + Viola tricolor | 2 | 5.78 ± 0.58 | 3.2 | 5.79 ± 1.07 | 3.1 |
| Tagetes patula + Viola tricolor | 5 | 5.37 ± 0.73 | 10.1 | 5.73 ± 0.51 | 4.1 |

* significant differences (time),
** significant differences (concentration), R30 - value of the change in number S. aureus after 30 min, R120 - value of the change in the number of S. aureus after 120 min.
Table 2. Effect of interaction time of 2% and 5% macerates on survival rate of S. aureus ATCC 25923.

| Type of flowers | Zone diameter [mm] | Correlation equations | Coefficient of determination $R^2$ |
|-----------------|-------------------|------------------------|-----------------------------------|
| Tagetes patula   | 2                 | $y = -0.32 + 6.22$     | 0.874                             |
| Tagetes patula   | 5                 | $y = -0.405 + 6.25$    | 0.777                             |
| Viola tricolor   | 2                 | $y = -0.615 + 6.64$    | 0.976                             |
| Viola tricolor   | 5                 | $y = 0.56x^2 - 2.16x + 7.57$ | 1                                 |
| Tagetes patula + Viola tricolor | 2 | $y = -0.09x + 6.026$ | 0.708                             |
| Tagetes patula + Viola tricolor | 5 | $y = -0.12x + 5.93$ $y = 0.48x^2 - 2.04x + 7.53$ | 0.157 1 |

x - interaction time between S. aureus and flower macerate.

There are no data in the literature on the biostatic properties of edible flower macerates or their juices, assessed by the diffusion disc method. Most studies focused on water extracts, ethanol extracts, methanol-water extracts or extracts in which the solvents are hexane or ethyl acetate. Antibacterial activity was assessed using the disc diffusion method. According to some authors, the antimicrobial activity of the aqueous extract assessed in relation to S. aureus to the aqueous extract was noted. The maximum inhibition of staphylococci was obtained using a 1-µg/mL solution of Viola (Gautam and Kumar, 2017, Muhammad et al., 2012). Studies using the species Viola betonicifolia reported no inhibition of S. aureus for both aqueous flower extracts and methanol solutions or with ethyl acetate or hexane. Antibacterial activity was assessed using the disc diffusion method. According to some authors, the antimicrobial activity of the aqueous Viola odorata extract assessed in relation to S. aureus is as high as 41.8 ± 2.4%. Also, a high sensitivity of S. aureus to the aqueous extract was noted. The maximum inhibition of staphylococci was obtained using a 1-µg/mL solution of Viola (Gautam and Kumar, 2017).

When using the oil obtained from Viola partitina, the inhibition zone of S. aureus was 6.62 mm at a concentration of 0.250 mg/mL (Yousuf et al., 2021). Koshkam et al. (2016) obtained an inhibition zone of 10 mm using a butanol extract of proteins from V. tricolor flowers. This degree of biostatic activity of Viola extracts obtained by the disc diffusion method is similar to that obtained in our experiment. Jha et al. (2012) indicate that water extracts from flowers of the genus Viola spp. do not show any ability to inhibit Staphylococcus aureus. This confirms our theory that it is necessary to study the effects of all petal components in unprocessed macerated tissue or sap. The use of a methanol-water extract of macerated flowers and leaves derived from T. patula resulted in an inhibition zone comparable to that observed in our experiment (Latiffian et al., 2021). The high concentration of methanolic flower extract caused an inhibition zone of up to 12 mm, whereas plant leaf extracts resulted in a zone of up to 10 mm. Faizi et al. (2008) showed that the methanol-water extract of T. patula flowers caused the formation of an inhibition zone with a diameter of 10–14 mm, whereas the patuletin component extracted from these flowers produced a zone of 8–23 mm. These authors concluded that T. patula flowers constitute a good mixture of components with antimicrobial properties. Comparable results were obtained by Jain et al. (2012), who studied water extracts of Tagetes erecta and T. patula flowers. The authors observed a high antimicrobial activity against S. aureus, with an inhibition zone of 26 mm. The diameters of the staphylococcal inhibition zones obtained with the use Tagetes petal juice obtained in our experiment were about 54% smaller compared to those obtained by Jain et al. (2012).

4. Conclusions

The reduction of staphylococci number by macerates of the petals and their mixtures did not exceed 11% of the initial population value. Low inhibition of S. aureus ATCC 25923 was found for the macerate and T. patula sap, using both methods. The diffusion disc method revealed the synergistic effect of the petals of both species on S. aureus cells ATCC25923. The contact of the Viola sap with the outer coatings of the staphylococcal cells in the mixture (variant C) indicates easier penetration and a higher degree of inhibition of bacteria compared to Tagetes. Our results lead us to infer that salads containing a mixture of Viola and Tagetes flower petals show higher biostatic activity in relation to staphylococcal cells compared to those only containing the flowers of one species.

Declarations

Author contribution statement

Izabela Steinka: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Jadwiga Stankiewicz; Anita Maria Kukulowicz.; Aleksandra Wilczyńska: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interest’s statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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