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Research Article

Antimicrobial Effect of Medicinal Plants on Microbiological Quality of Grape Juice

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Abstract
The safety of plant-based food of plant origin is a priority for producers and consumers. The biological value of food products enriched with herbal ingredients is getting more popular among consumers. The present study was aimed to evaluate microbiological quality of grape juice enriched with medicinal plants. There were two varieties of grapes - Welschriesling and Cabernet Sauvignon and six species of medicinal plants used for the experiment: Calendula officinalis L., Ginkgo biloba, Thymus serpyllum, Matricaria recutita, Salvia officinalis L., and Mentha aquatica var. citrata. A total of 14 samples of juice were prepared and two of them were used as controls and 12 samples were treated with medicinal plants. Total microbial count, coliforms, lactic acid bacteria and yeasts and microscopic fungi for testing the microbiological quality were detected. All the samples were negative for lactic acid bacteria. There were neither coliform bacteria nor yeast and fibrous microscopic fungi present in some of the treated samples apart from the control samples. Coliform bacteria of 1 log cfu/mL were detected during the first testing and identified Proteus vulgaris in the control sample of grape juice of the variety Welschriesling. The yeast of the genus Candida was present in 2 log cfu/mL and it was detected during first and second testing in the control sample of Cabernet Sauvignon, unlike the other samples. The sample of grape juice with the addition of sage had the most significant inhibitory effect on total count of bacteria. The most common microorganisms in all samples were Bacillus cereus and Acinetobacter johnsonii, however, those microorganisms were absent during second testing due to the action of naturally occurring antimicrobials in the grapes.

Keywords: grape, grape juice, medicinal plants, medicinal plants, antimicrobial activity.

Introduction

Grapes, lat. *Vitis vinifera* belong to the important crops that are widely grown all around the world. It can be consumed directly (berries), dried to raisins, processed into must, juice or wine or other products [1]. Grape juices can be prepared from any grape variety (white or blue) as soon as they are properly ripened. Many observational studies suggest that intake of pure (100%) grape juice may reduce the risk of hypertension, cardiovascular disease, and diabetes mellitus, suggesting the importance of these drinks for maintaining health [2]. Consumption of grape juice gradually increases due to factors such as

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characteristic taste, aroma, color or refreshment. In addition, grape juice is gaining popularity due to the functional properties associated with consumer health, which are mainly related to phenolic compounds [3].

Medicinal plants are considered to be valuable and very useful plants. They are defined as higher-growing or cultivated plants which contain various physiologically active substances in their parts (roots, leaves, flowers). Medicinal plants are called medicinal plants because they have a therapeutic effect on the human body. However, some of them may also act as a poison when used in high concentrations [4]. It has been found that the antimicrobial properties of medicinal plants can be used to control the growth of certain pathogenic bacteria and fungi as well as to reduce food spoilage. Thus, they are considered as a good substitute for artificial antimicrobials used in food production [5]. Over the last few decades, scientific studies have shown that plant and herbal extracts can be used to some extent as natural preservatives. In addition, by applying various herbs, it is possible to develop new food products with new and interesting flavor variants [6]. The anti-inflammatory effect of *Calendula officinalis* L. extracts is mainly due to the presence of fatty acid esters with pentacyclic triterpenols. These ingredients, together with other active ingredients, have other healing effects, including antibacterial, anticancer and immune activities, and they also improve gastrointestinal function [7]. Secondary metabolites or active ingredients of *Ginkgo biloba* have been shown in some studies to increase memory and promote a beneficial therapeutic effect on neurodegenerative diseases [8]. *Ginkgo biloba* extracts have been used for more than 5,000 years to treat infectious lung diseases such as bronchitis, as well as cardiovascular disease in China [9]. *Thymus serpyllum* is traditionally used to treat respiratory, digestive and urological problems. Highly concentrated infusion has been shown to be useful in diluting bronchitis secretion or even in preventing hair loss. The aqueous extract of the mother's tube has been shown to have the ability to capture nitric oxide [10]. In addition to antibacterial effects, it also acts as a natural preservative and an anti-aging agent [11]. Due to its pharmacological and pharmaceutical properties, chamomile has a high economic value. It is used in both fresh and dried form in food industry. Fresh flower heads are added to salads or teas. Dried flowers are used in the production of herbal teas or in bakeries [12]. Historically, sage was used in ancient Egypt to increase fertility in women, in the treatment of menopausal and menstrual problems, or in infertility [13]. Lemon mint has been used to eliminate or alleviate headaches, indigestion, muscle aches, rheumatism, convulsions, respiratory distress, irritated skin, as an ingredient in mouthwashes, or to partially fight fungi and viruses in folk medicine [14]. It is also used as an aromatic ingredient in confectionery, beverages, pharmaceuticals, cosmetics, perfumery resp. other industrial products [15].

The aim of the present study was to evaluate microbiological quality of grape juice enriched with medicinal plants.

Material and Methods

There were two types of grapes used in the testing - white grapes (variety Welschriesling) and blue grapes (variety Cabernet Sauvignon), which were obtained from SPU from the Department of Fruits, Viticulture and Enology.

Preparation of grape juice samples

*T*he juice from the grapes was prepared by using the following grape dressing procedures: harvesting, destemming, maceration, pressing, sludge removal and pasteurization. Grapes were collected from the Welschriesling and the Cabernet Sauvignon variety on October, 14th 2020 in the morning in warm and sunny weather. Grapes were harvested manually in crates with a volume of 25 kg and they were immediately transported the grapes to the AgroBioTech research center to the laboratory of beverages intended for processing in order to prevent possible steaming of the grapes after harvesting. The sugar content of the Welschriesling grape was immediately after harvest 19.87 °Bx and the Cabernet Sauvignon 16.35 °Bx. Then the grapes were poured into a central component for mechanical destemming, where the chunks were separated from the must and berries. The grape mash maceration lasted one hour in closed containers with a volume of 100 L. The pressing was performed in a gentle way using the maximum pressing pressure - 0.1 MPa. Discontinuous hydrolis was used for pressing. The aim of pressing at a lower pressure is to eliminate damage to the grape seeds and the subsequent transfer of immature phenolic substances to the pressed must itself. In the case of pressing at a pressure higher than 0.15 MPa, undesirable substances can pass into the pressed must. These substances could thus negatively affect the future character of the must and its sensory properties. Then the grape was pumped must into a sludge tank using a pump. The static sludge removal happened without the use of oenological auxiliaries there. Subsequently, both experimental musts were pasteurized in order to eliminate possible contamination by unwanted

microcenosis, in particular by fermentation of the must under the influence of wild yeasts.

Preparation of medicinal plants
Plants were picked in Slovakia 48°56'05.8"N, 18°07'32.9"E. There were approximately 20 g of dried plants of *Calendula officinalis* L., *Ginkgo biloba*, *Thymus serpyllum, Matricaria recutita, Salvia officinalis* L., and *Mentha aquatica* var. *citrata* evaluated. The extracts were prepared in a ratio of 1:10 and thus we mixed 10 g of the medicinal plant with 100 ml of ethanol. The herbal mixture prepared in this way was left to infuse for 2 weeks. The herbal mixture was treated by evaporation on a vacuum evaporator and subsequent dissolution in distilled water after these 2 weeks.

Preparation of grape juice enriched with selected medicinal plants
There were used prepared samples of musts from the varieties of Welschriesling and Cabernet Sauvignon in a volume of 200 ml and extracts from selected medicinal plants in a volume of 20 ml for the preparation of grape juices enriched with medicinal plants. Therefore the mixtures of grape juices with herbal extracts in a ratio of 1:10 were prepared. The samples prepared in this way were used to monitor the microbiological quality.

Microbiological analyses
There were total count of microorganisms, microscopic filamentous fungi, coliform bacteria and lactic acid bacteria evaluated in our study [16]. There were also total count of microorganisms on plate count (PCA, Oxoid, UK) at 30 °C during 48 h, microscopic filamentous fungi on Malt extract agar (MEA, Oxoid, UK) at 25°C for 5 days, coliform bacteria on violet red bile agar (VRBL, Oxoid, UK) at 37°C for 24 h and lactic acid bacteria on Main Rogosa agar (MRS, Oxoid, UK) at 30°C for 48 h evaluated. Typical microbial colonies were identified with MALDI-TOF MS Biotyper (Bruker Daltonics, Germany) [17].

Statistical analyses
The basic statistical method using Microsoft Office Excel (arithmetic mean calculation of 3 measurements) and the statistical program GraphPad Prism 6 (GraphPad Software, San Diego, USA), where we used two-factor analysis of variance ANOVA (Dunnett test) at 4 levels of significance P <0.01, P <0.001, P <0.0001 and P <0.00001 for evaluation of individual parameters and test data was used.

Results and Discussion
Coliform bacteria were able to grow only in the control sample, the added medicinal plants to the grape juice reliably destroyed the number of these bacteria (Table 1).

Table 1.
Number of microorganisms isolated from white grape juice with medicinal plants in log cfu/mL in first day of storage

Sample	TCM	MFF	CB	LAB
CW	2.89	0.00	1.00	0.00
W1	1.45**	0.00	0.00	0.00
W2	1.89*	0.00	0.00	0.00
W3	1.85*	0.00	0.00	0.00
W4	1.65**	0.00	0.00	0.00
W5	1.36**	0.00	0.00	0.00
W6	1.78**	0.00	0.00	0.00

Legend: CW - white grape juice control sample, W1 - white grape juice with the addition of *Calendula officinalis* L.,
W2 - white grape juice with the addition of *Ginkgo biloba*, W3 - white grape juice with the addition of *Thymus serpyllum*,
W4 - white grape juice with the addition of *Matricaria recutita*,
W5 - white grape juice with the addition of *Salvia officinalis* L.,
W6 - white grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * P <0.01, ** P <0.001, *** P <0.0001,
TCM – total count of microorganisms, MFF – microscopic filamentous fungi, CB – coliform bacteria, L- lactic acid bacteria

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The highest inhibitory effect against Total count of microorganisms (TCM) was demonstrated by the addition of *Salvia officinalis* L., where the microorganisms were able to grow in an amount of 1.36 log cfu/mL. The second most significant effect was *manifested Calendula officinalis* L., with the number of grown bacteria 1.45 log cfu/mL. The highest number of microorganisms in samples with the addition of medicinal plants was found in samples containing *Ginkgo biloba* and

Thymus serpyllum. Despite this amount, they suppressed a relatively high number of microorganisms compared to the control sample by up to 64.71% at the level of evidence P <0.01. The inhibitory effects of *Mentha aquatica* var. *citrata* and *Matricaria recutita* against microorganisms were moderately effective compared to other treated juices and the cfu values did not exceed of 1.78 log cfu/mL. The other bacteria of white grape juice were not isolated.

Coliform bacteria were completely inhibited in the control sample, presumably by the action of phenolic compounds after one week and repeated measurements (Table 2).

Table 2.

Number of microorganisms isolated from white grape juice with medicinal plants in log cfu/mL after first week of storage

Sample	TCM	MFF	CB	LAB
CW	3.25	0.00	0.00	0.00
W1	1.31***	0.00	0.00	0.00
W2	1.76**	0.00	0.00	0.00
W3	1.72**	0.00	0.00	0.00
W4	1.54***	0.00	0.00	0.00
W5	1.22***	0.00	0.00	0.00
W6	1.62**	0.00	0.00	0.00

Legend: CW - white grape juice control sample, W1 - white grape juice with the addition of *Calendula officinalis* L., W2 - white grape juice with the addition of *Ginkgo biloba*, W3 - white grape juice with the addition of *Thymus serpyllum*, W4 - white grape juice with the addition of *Matricaria recutita*, W5 - white grape juice with the addition of *Salvia officinalis* L., W6 - white grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * P <0.01, ** P <0.001, *** P <0.0001, TCM – total count of microorganisms, MFF – microscopic filamentous fungi, CB – coliform bacteria, L- lactic acid bacteria.

On the contrary, the amount of TCM increased by 11.08%. In addition, the numbers of microorganisms in the juices with the addition of medicinal plants have been significantly reduced, indicating a high potential for the protection of juices in the long term. There were also significant differences in TCM between the control sample and the treated plant samples at the level of evidence P <0.001 and P <0.0001. The highest inhibitory effect was achieved by *Salvia officinalis* L., up to 62.46% compared to the control sample. There was found a significant increasing inhibitory effect of *Mentha aquatica* var. *citrata* of all treated grape juice samples (8.99%) in comparison with the results achieved after one week.

Even in the case of the samples of white grape juice, there was a certain amount of microbiota in them, which we were able to isolate and identify (Table 3).

Table 3.

Isolated species from white grape juice with medicinal plants

Sample	1. day	1. week
CW	*Acinetobacter johnsonii, bacillus cereus, Bacillus megatherium, Pantoea agglomerans, Proteus vulgaris*	*Bacillus simplex, Bacilus megatherium*
W1	*Bacillus cereus*	*Bacillus cereus*
W2	*Bacillus megatherium, Bacillus simplex*	*Bacillus megatherium*
W3	*Bacillus* spp*., Bacillus megatherium, Proteus vulgaris*	*Bacillus simplex, Bacillus megatherium*
W4	*Acinetobacter johnsonii, Bacillus cereus, Bacillus megatherium, Pseudomonas mandelii*	*Bacillus megatherium, Bacillus cereus*
W5	*Bacillus megatherium, Bacillus simplex*	*Bacillus simplex, Bacillum megatherium*
W6	*Bacillus flexus, Bacillus simplex*	*Bacillus simplex, Bacillus megatherium*

Legend: CW - white grape juice control sample, W1 - white grape juice with the addition of *Calendula officinalis* L., W2 - white grape juice with the addition of *Ginkgo biloba*, W3 - white grape juice with the addition of *Thymus serpyllum*, W4 - white grape juice with the addition of *Matricaria recutita*, W5 - white grape juice with the addition of *Salvia officinalis* L., W6 - white grape juice with the addition of *Mentha aquatica* var. *citrata.*

Bacillus and *Acinetobacter johnsonii* species were most frequently found in white grape juice samples. In addition, coliform bacteria, namely *Proteus vulgaris*, which probably entered the juice during its handling, were also present in the control sample. These bacteria were reliably destroyed after a week of storage, firstly by the phenolic compounds contained in the grape juice.

It is obvious from the obtained results that the addition of medicinal plants had a positive effect on the inhibition of microorganisms.

It was also found out that none of the samples contained coliform bacteria, microscopic filamentous fungi or lactic acid bacteria after testing the samples of blue grape juice (Table 4).

Table 4.

Number of microorganisms isolated from blue grape juice with medicinal plants in log cfu/mL in first day of storage

Sample	TCM	MFF	CB	LAB
CB	2.36	2.00	0.00	0.00
B1	1.63	0.00	0.00	0.00
B2	1.42	0.00	0.00	0.00
B3	1.26	0.00	0.00	0.00
B4	1.65	0.00	0.00	0.00
B5	1.49	0.00	0.00	0.00
B6	1.56	0.00	0.00	0.00

Legend: CB - blue grape juice control sample, B1 - blue grape juice with the addition of *Calendula officinalis* L.,
B2 - blue grape juice with the addition of *Ginkgo biloba*, B3 – blue grape juice with the addition of *Thymus serpyllum*,
B4 - blue grape juice with the addition of *Matricaria recutita*,
B5 - blue grape juice with the addition of *Salvia officinalis* L.,
B6 - blue grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * P <0.01, ** P <0.001, *** P <0.0001, TCM – total count of microorganisms, MFF – microscopic filamentous fungi,
CB – coliform bacteria, L- lactic acid bacteria.

The control sample contained relatively high yeast values, specifically in the number 2.00 log cfu/mL. Medicinal plant-added juices did not show a statistically significant difference compared to the control sample when tested for TCM. Nevertheless, they inhibited a number of microorganisms and this increase in the dose of medicinal plants would exacerbate this effect. The *Thymus serpyllum* had the best effect on microorganisms, where the TCM did not exceed a value of 1.26 log cfu/mL. Juice samples with the addition of *Salvia officinalis* L. and *Ginkgo biloba* reached mean values of the effects of inhibition of microorganisms. The weakest of the juices treated with medicinal plants was the *Calendula officinalis* L. and *Matricaria recutita*, where the percentage of inhibition compared to the control sample was 30%. The number of yeasts did not change after one week in the control sample of blue grape juice (Table 5).

Table 5.

Number of microorganisms isolated from blue grape juice with medicinal plants in log cfu/mL after first week of storage

Sample	TCM	MFF	CB	LAB
CB	3.21	2.00	0.00	0.00
B1	1.57*	0.00	0.00	0.00
B2	1.34*	0.00	0.00	0.00
B3	1.12*	0.00	0.00	0.00
B4	1.52*	0.00	0.00	0.00
B5	1.38*	0.00	0.00	0.00
B6	1.35*	0.00	0.00	0.00

Legend: CB - blue grape juice control sample, B1 - blue grape juice with the addition of *Calendula officinalis* L.,
B2 - blue grape juice with the addition of *Ginkgo biloba*, B3 – blue grape juice with the addition of *Thymus serpyllum*,
B4 - blue grape juice with the addition of *Matricaria recutita*, B5 - blue grape juice with the addition of *Salvia officinalis* L.,
B6 - blue grape juice with the addition of *Mentha aquatica* var. *citrata*, significance to the control sample at the level of evidence * P <0.01, ** P <0.001, *** P <0.0001, TCM – total count of microorganisms,
MFF – microscopic filamentous fungi, CB – coliform bacteria, L- lactic acid bacteria.

On the contrary, TCM increased by 26.48%. Despite the initial unproven statistical significance of the testing of blue grape juice in the first week, the antimicrobial effect of the medicinal plants increased after one week of storage to a level of evidence P<0.01. The *Thymus serpyllum* mouth was similarly as in the first test, the most effective against microorganisms compared with other treated juices. On the contrary, the weakest effect was again manifested by *Calendula officinalis* L. and *Matricaria recutita*. The most significant improvement in the inhibitory effect compared to the first test was achieved by *Mentha aquatica* var. *citrata*, whose intensity against microorganisms increased by 13.46%. *Ginkgo biloba* increased its antimicrobial effect the least, while the number of TCM decreased from 1.42 log cfu/mL to 1.34 log cfu/mL in comparison with other juices enriched with medicinal plants. There was a moderate increase in inhibition achieved by *Salvia officinalis*.

The microbiota of grape juice consists of different types of microorganisms which are naturally present in it or enter it in many different ways, for example during its handling (Table 6).

Table 6.

Isolated species from blue grape juice with medicinal plants

Sample	1. day	1. week
CB	*Bacillus megatherium, Candida albicans, Pseudomonas alcaligenes*	*Acinetobacter johnsonii, Bacillus cereus, Candida* spp.
B1	*Bacillus simplex*	*Bacillus simplex, Bacillus megatherium*
B2	*Acinetobacter junii, Bacillus megatherium, Bacillus simplex, Pantotea agglomerans*	*Bacillus megatherium*
B3	*Baccilus megatherium, Pseudomonas alcaligenes*	*Bacillus megatherium, Pseudomonas stutzeri*
B4	*Acinetobacter johnsonii, Bacillus cereus, Pseudomonas* spp.	*Acinetobacter johnsonii, Bacillus cereus, Pseudomonas* spp.
B5	*Bacillus megatherium, Bacillus subtilis* supsp. *Subtilis, Bacillus simplex*	*Bacillus megatherium*
B6	*Bacillus simplex*	*Bacillus simplex*

Legend: CB - blue grape juice control sample, B1 - blue grape juice with the addition of *Calendula officinalis* L., B2 - blue grape juice with the addition of *Ginkgo biloba*, B3 – blue grape juice with the addition of *Thymus serpyllum*, B4 - blue grape juice with the addition of *Matricaria recutita*, B5 - blue grape juice with the addition of *Salvia officinalis* L., B6 - blue grape juice with the addition of *Mentha aquatica* var. *citrata.*

There was a certain amount of microbiota in the samples of grape juice, which was possible to isolate and identify. *Bacillus* and *Acinetobacter johnsonii* species were most frequently found in white grape juice samples. In addition, coliform bacteria, namely *Proteus vulgaris*, which probably entered the juice during its handling, were also present in the control sample. These bacteria were reliably destroyed after a week of storage, firstly by the phenolic compounds contained in the grape juice. The obtained results showed that the addition of medicinal plants had a positive effect on the inhibition of microorganisms.

Oranusi et al. [18] examined the microbiological and chemical quality of commercially packaged fruit juices sold in Nigeria. They used a total of 30 samples of fruit juices based on general popularity - orange, lemon, pineapple, punch and guava juice. Samples were stored at 4 °C prior to testing. Samples were removed from the refrigerator for a period of time to adjust to ambient temperature (approximately 28±2°C) for microbiological analysis. There were found coliform bacteria in lemon juice $2.8x10^4$ cfu/ml), pineapple juice ($6.0x10^4$ cfu/mL) and guava juice ($1.1x10^4$ cfu/mL) after appropriate cultivation. Microscopic fungi or yeast were found in all samples in an amount from $1.0x10^4$ cfu/mL to $4.3x10^4$ cfu/mL. There were found aerobic mesophilic bacteria as well as yeast and microscopic filamentous fungi in all analysed samples in different number. The lowest number of aerobic mesophilic bacteria was found in lemon juices ($1.1x10^4$ cfu/mL) and the most significant amount was represented in pineapple juices (up to $9.6x10^4$ cfu/mL). They performed worse results compared to our samples, because no coliform bacteria or microscopic filamentous fungi and yeast were present in our samples except for the control samples. Only the control sample of white grapes contained coliform bacteria in the amount of 1.00 cfu/mL and the control sample of blue grapes contained yeast in the amount of 2.00 cfu/mL. In addition, Oranusi et al. [18] isolated certain species of microorganisms, *Bacillus* spp.

and *Staphylococcus aureus*. *Bacillus* spp. was also the most common bacterium in our samples. It seems from the obtained results of microbiological testing that the studied samples achieved significantly better microbiological quality.

Bello et al. [19] were focused on the microbiological quality of fruit juices in their study, using 120 samples of fruit juices for analysis. The tested juices were from 5 types of fruit - avocado, papaya, pineapple, grapes and orange. There were used 24 samples of juice from each species in a volume of 25 ml. The grape juice samples contained $1.9x10^4$ cfu/mL coliform bacteria, $1.3x10^4$ cfu/mL staphylococci, 1.9x104 cfu/mL yeast and 2.6 $x10^4$ cfu/mL microscopic filamentous fungi. Specifically, *Bacillus cereus, Staphylococcus aureus, Saccharomyces cerevisiae* and *Aspergillus niger* were the most isolated microorganisms found in the juices. Bello et al. [19] samples generally found more microorganisms in comparison with our samples, and thus our samples had better microbiologically quality, mainly samples treated with selected medicinal plants.

Conclusions

There are very few data focused on the testing of grape juice with the addition of medicinal plants. The aim of our work was the microbiological quality of grape juice with the addition of various herbs. In the first and second measurements of grape juice samples of the Welschriesling variety, the sample of grape juice with the addition of *Salvia officinalis* L. had the most significant inhibitory effect on TCM. It most strongly inhibited the growth of TCM in the grape juice of the Cabernet Sauvignon variety with addition of *Thymus serpyllum*, and, conversely, the most resistant microorganisms were manifested in addition to the control sample in the sample with the addition of *Calendula officinalis* L.

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