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When silver flakes, strawberry tree and rose water combines, what happens to two Gram positive and two Gram negative pathogenic bacteria?

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Abstract

An antimicrobial is defined as any of a diverse variety of chemical and physical agent, capable of destroying microorganisms or to prevent their development in natural and synthetic way. They can be either produced by microorganisms or chemical substances derived from plants if they are said to be natural. A study was designed to mix silver flakes with rose water (Eau de rose) and strawberry tree fruit (*Arbutus unedo*) in order to produce an antimicrobial complex. The studies were performed on the determination of its antimicrobial properties. The effect of this complex was tested on two Gram positive and two Gram negative pathogenic bacteria, therefore the stability of different pH and temperature on the complex was analyzed. It was found to be effective at the lowest pH and temperature values on all bacteria investigated in our study. Moreover the control and the complex was found to be significantly different from the statistical point of view.

Keywords: Strawberry tree fruit, Silver flakes, Rose water, Antimicrobial

1. Introduction

An antimicrobial is defined as a substance that inhibits the growth of or kills microorganisms. These substances may be not only in a form of a chemical compound, but also it can exist as a physical agent. They are capable of interacting with the growth and reproduction of a wide range of organisms such as bacteria, parasites, viruses and molds etc. (El Fane et al., 2017). some elements existed in nature, such as minerals like copper, silver, bromine etc. show antibacterial properties when present in high concentrations. These agents find their use in many fields such as veterinary, medicine, home appliances etc. The thorough application of environments containing pathogenic microorganisms by intensive disinfectant nanomaterials has been proposed for elimination of the epidemics. A typical example is the silver nanoparticles (Ag-NPs) with unique properties of intensive antimicrobial action have gained much interest from the scientific and technologic communities to develop nanosilver-based disinfectant products (Tran et al., 2013).

Since one of the main nanomaterial preferred in various industries is silver, its easy incorporation into other substances or its use for coating allows silver an available element for medical purposes by its antibacterial and disinfection properties. Nanosilver is used in the production of dressings, surgical instruments or implants. It is also applicable in environmental engineering, for example, water treatment and purification processes. It finds its application in the production of textiles, underwear, and cosmetics (Chojniak et al., 2016). The remnants of silver in the industry was also said to be a problem from the environmental point of view. Due to this reason, thin metallic silver flakes were screened out for their antimicrobial characteristics in aqueous phase. The flakes used in that study were provided from a company producing silver films, as waste materials. By this way the cost of silver will be kept low in screening assays. Their easy application can allow them to be involved in filters, devices or formulations without using any supporting material. This is the first instance that this type of silver is used as antimicrobial agent (Anzano et al., 2011).

Strawberry tree (*Arbutus unedo* L.) is a typical example to these kind of plants; its leaves and fruits are used in folk medicine for treating inflammation, hypertension, and diabetes (Jurica et al., 2017). It contains many

different antioxidative compounds and display antimicrobial property(Ülgen, 2013). Other than medicine, in food production and nutritional sciences, the bioactive compounds not only contain antioxidative activity, but also the reactive oxygen species and halogenated hydrocarbons generating free radicals can be neutralized by these biological molecules, avoiding serious pathological disorders (Gupta et al., 1992). The roots and bark of strawberry tree is considered as valuable in folk medicine like gastrointestinal, urological and dermatological disorders; via decoction of the roots the "drug" is ready to drink. Other than these findings, the strawberry tree is not yet to be exactly discovered, extremely due to the high heterogenic flora. The content of biologically active substances present in the different parts of this tree is related with its medicinal and therapeutical performance. The composition of fruits, bark and roots, as well as its biological activity and positive healthcare effects of extracts of the strawberry tree was characterized by Alarcão-e-Silva and coworkers in 2001 (Oliveira et al., 2011).

Rose water is believed to stop and cure infectious disorders by its antiseptic characteristics. Due to this property, rose water is frequently preferred in various natural and medical treatments. A common eye disorder, conjunctivitis was found to be treated by rose water, in eye drop form depending on its antiseptic and analgesic characteristics, reported by a case study (URL 1). As both an eyewashing agent and a moisturizer, rose water displays its antiseptic characteristics. By using the bioactive substances, antimicrobial and antioxidative action of rose water is maintained, attributing to the presence of flavonoids and the phenolics (Bahl et al., 2016). In addition, roses are greatly appreciated when the inimitable aroma, extensive uses, and their beauty. The different damask rose products such as oil, water, concrete, and gulkand, its dried petals are also produced for protective healthcare. Roses have solid (stearoptene) and liquid (oleoptene) components, as well as they contain high amount of corilagin and tellimagrandin. In general rose water showed antimicrobial activity in fruit juices too (Yurdugul et al., 2016).

Listeriosis is the main disease caused by this bacteria and especially newborns, the elder, patients with impaired immune system and women who has pregnancy are mainly affected, as well as healthy individuals may suffer from this disorder. The symptoms of invasive listeriosis are listed as abortion in pregnancy, septic infection and meningoencephalitis. Moreover, it can show febrile gastroenteritis. Most species of vertebrates are reported to be affected by this bacteria. *S. aureus* is a gram positive bacteria, sized in regular cocci, linked together in clusters such as a chain. In some conditions such as an aged culture, a resolved lesion, and if there is an antibiotic, the cellular structure is mostly reported to be varied in size, and interestingly most cultures are no longer to be a gram positive bacteria. *S. aureus* contains teichoic acid, showing antigenic and relatively specific for this bacteria such as a regular gram positive peptidoglycan including a ribitol.

Acinetobacter baumannii is a gram negative, non-motile, obligate aerobic coccobacillus having effective virulence factors, including its attachment to and resisting on solid and dry places, its effort to supply vital elements like iron, the attachment to and sequential epithelial cell destruction, and the productivity of gelatinases and proteinases that can give harm to related tissues in some strains. *A. baumannii* can be able to live in the skin of patients or even healthy people without causing any disease. *A. baumannii* has commonly defined as an important nosocomial pathogen, being easily resistant to common antibiotics abundant in the market (Ryan and Ray, 2004, Boucher and Talbot, 2009).

Klebsiella pneumoniae is one of the most prevalent gram negative bacteria encountered by physicians in the world. Likewise Staphylococcus and Acinetobacter, it is a widespread nosocomially acquired microorganism, leading to infectious problems of urinary tract, hospital acquired pneumonia, and intraabdominal infections. *K. pneumoniae* is also reported to be a potential society acquired bacteria. An internationally collaborated research showed the geographic differences and tendencies of society acquired Klebsiella infection, indeed *K. pneumoniae* has been reported as a bacteria which can cause pulmonary infections (Ryan and Ray, 2004). In this study we aimed to observe the effect of the combination of the silver flakes, strawberry tree and rose water to kill or inhibit two gram positive and two gram negative pathogenic bacteria. Subsequently following the preparation of the combined substance, the antimicrobial characteristics was determined. The potential combined agent might be available to be used in the structure of a cleaning agent in future or in a food/beverage, depending upon its possible antimicrobial activity.

2. Material and Methods

2.1. Materials

2.1.1.Silver flakes

Silver flakes were obtained from a local vendor in Bolu, TURKEY.

2.1.2. Rose water

Rose water (Eau de rose) was purchased from Gülbirlik A.Ş., Isparta, TURKEY.

2.1.3.Strawberry tree (Arbutus unedo L.)

Arbutus unedo L. (Strawberry tree) fruits were harvested from the environs of Akçakoca, Düzce, and Karadeniz Ereğli, Zonguldak, TURKEY.

2.1.4.The Bacteria

Two reference bacteria, tested in this study were recruited from the microbiology laboratory of the hospital of Abant Izzet Baysal University, Faculty of Medicine, Department of Microbiology, Bolu, TURKEY. They were listed as follows: *A. baumannii* ATCC 12365 and *K. pneumoniae* ATCC 12528. *L. monocytogenes* and *S.aureus* were provided as a generous gift from Food Engineering Department, Faculty of Engineering of Abant Izzet BAYSAL University.

2.2.Method

2.2.1. The freeze-drying process of the strawberry tree fruits

Strawberry tree fruits, kept frozen at -80° C was lyophilized by a freeze-drier (Christ, Germany) with a temperature of -57º C for 24 hours.

2.2.2.The determination of the best effective dose

The dose determination was carried out by Kirby-Bauer disc diffusion method. For this purpose, 5, 10, 20 and 50 µL/mL of the rose water (vol/vol) was used against the microorganisms mentioned in Section 3.1.4. according to the method of Kırmusaoğlu, 2010; with modified amounts. From 67 mg/mL stock solution of the rehydrated strawberry tree fruit, 10, 20, 30 and 40 µL was tested on the reference bacteria and the other species. The silver flakes was tested as 10, 20, 30 and 40 mg/mL, on the reference organisms and the other bacteria according to the method of Anzano et al.(2011); with slightly modified amounts.

Agar disc diffusion (Kirby-Bauer) method

All bacteria were first inoculated into 10 mL Mueller-Hinton broth (MHB) containing test tubes by 1-2 sterile loops and adjusted to 0.5 McFarland (approximately 1.5×10^8 cfu/mL) by a McFarland densitometer (Biosan, Model Den-1, Latvia). From this stock, individually, a sterile cotton swab was dipped into each of the standardized bacteria and it was used to streak the entire surface of the plates containing Mueller-Hinton agar (MHA) in triplicates. Sterile filter paper discs (Glass Microfiber filters, Whatman, 6 *mm* in diameter) were treated with the appropriate amounts of rose water, rehydrated strawberry tree fruit powder and silver flakes reported in Section 3.2.2, individually. These disc containing inoculated plates were incubated at 37º C for a period of 24-48 hours and the diameters (*mm*) of the inhibition zones were measured after incubation(Bauer et al., 1966).

Testing the antimicrobial activity of the combination

The antimicrobial activity of the strains *A. baumannii* ATCC 12365, *K. pneumoniae* ATCC 12528, *L.monocytogenes* and *S. aureus* were tested in this assay. For this purpose, individually, all bacteria were first cultured in a Mueller-Hinton Broth (MHB) (Merck, Germany). The reference bacteria were adjusted to a concentration of approximately 1.5×10^8 CFU/mL (0.5 Mc Farland). Afterwards, for the preparation of 10 mL

bacterial suspension, 1 mL bacteria were transferred to 9 mL sterile distilled water in control and homogenized by thorough vortexing, in combination, 1 mL bacteria was thoroughly homogenized with 1 mL antimicrobial combination (Table 3.2.1) and 8 mL sterile distilled water containing test tubes and serial dilutions were completed to 10^{-5}. From the dilutions of 10^{-3}, 10^{-4} and 10^{-5}, 0.1 mL was spreaded onto plate count agar (PCA) (Merck, Germany) and incubated at 37° C for 24-48 hours in triplicates. The composition of the combination medium and the final concentration of the ingredients during the inhibition tests was tabulated in Table 2. and 3 respectively. Susceptibility tests were made according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2007).

Table 1. The amount of bacteria in test tubes.

Bacteria	Gram staining	Amount of combination (mL)	Amount of sterile distilled water (mL)
1 mL *A. baumannii* ATCC 12365	G(-)	1	8
1 mL *K. pneumoniae* ATCC 12528	G(-)	1	8
1 mL *L.monocytogenes*	G(+)	1	8
1 mL *S. aureus*	G(+)	1	8
Control			
1 mL *A. baumannii* ATCC 12365	G(-)	-	9
1 mL *K. pneumoniae* ATCC 12528	G(-)	-	9
1 mL *L.monocytogenes*	G(+)	-	9
1 mL *S. aureus*	G(+)	-	9

2.2.5. The composition of the combination

Two different treatment groups (Group 1 and 2) including the control and the combination were prepared according to the formula tabulated in Table 2. The main components of the combination are freeze-dried strawberry tree fruits in freeze-dried form, rose water, silver flakes and sterile distilled water.

Table 2. The main composition of the combination and the control.

Control	Combination group
Sterile distilled water (100 mL)	6.7 g/100 mL rehydrated strawberry tree fruits powder (67 mg/mL).
	50 µL/mL rose water (5mL/100 mL)
	40 mg/100 mL silver flakes. (0.4 mg/mL)
	Completed to 100 mL sterile distilled water.

Table 3. The final concentrations of the combination and the control.

Components	**Group 1**			**Control**
Strawberry tree	$67x10^{-3}$mg/mL	$67x10^{-4}$ mg /mL	$67x10^{-5}$mg/mL	-
Rose water	$5x10^{-5}$ mL	$5x10^{-6}$ mL	$5x10^{-7}$ mL	-
Silver flakes	$4x10^{-4}$ mg/mL	$4x10^{-5}$ mg/mL	$4x10^{-6}$ mg/mL	-

The pH

The pH of the combination was analyzed by an ordinary pH meter (WTW Inolab 720, Germany) according to the reference method of AOAC 981.12 (AOAC, 1995).

The statistical analysis

All data were analyzed by using MedCalc Statistical Software (version 15.8). Prior to the ANOVA test, Levene's Test for Equality of Variances is performed. If the Levene test is positive (P<0.05) then the variances in the different groups are different (the groups are not homogeneous). If the ANOVA test is positive (P<0,05) then MedCalc performs a post hoc test (using Tukey-Kramer's method) for pairwise comparison of subgroups.

3. Results and Discussion

When the effect of the silver flakes was independently evaluated on *S. aureus, K. pneumoniae* ATCC 12528, *A. baumannii* ATCC 12365 and *L. monocytogenes* by using Kirby-Bauer disc diffusion method, the silver flakes was found to be effective on all species mentioned above (Figure 1).

Figure 1. The effect of silver flakes against *K. pneumoniae* ATCC 12528, tested by Kirby-Bauer disc diffusion method.

When the effect of the strawberry tree *(A. unedo)* was evaluated on *A. baumannii* ATCC 12365, *K. pneumoniae* ATCC 12528, *L. monocytogenes,* and *S. aureus* by using Kirby-Bauer disc diffusion method; independently, the rehydrated strawberry tree fruit was found to be effective on all bacteria mentioned above (**Figure 2**).

Figure 2. The effect of rehydrated strawberry tree fruit *(A. unedo*) against *S. aureus* tested by Kirby-Bauer disc diffusion method.

50 µLs of rose water was found to be the best dose, only effective on *Staphylococcus aureus* by using Kirby-Bauer disc diffusion method. As this was the only species found to be inhibited by rose water in our study, almost similar findings were observed in 9 MSSA, 4 MRSA, 13 MSSE and 15 MRSE strains studied between the range of 0.1-1 µL/mL by Kırmusaoğlu (2010). The best doses of the rose water, rehydrated strawberry tree fruit and the silver flakes which displayed inhibitory action in an individual manner were combined and the following results were obtained in Table 4.

Table 4. The mean and standard deviation values of the effect of the combination against the bacteria mentioned in Section 3.1.4. with respect to control.

	S.aureus	*A. baumannii* ATCC 12365	*K.pneumoniae* ATCC 12528	*L.monocytogenes*
The control	6.34±0.058[a]	6.16±0.131[a]	6.23±0.061[a]	5.95±0.095[a]
combination	5.87±0.029[b]	5.25±0.067[b]	5.84±0.076[b]	5.43±0.306[a]

[*]Means with the different letter indicates significant difference among the treatments ($p<0.05$).

3.1.The pH of the combination

The pH of the combination group was measured in order to evaluate the effect on different pathogenic bacteria. The pH of the combination was found to be 3.55 and the control was 7.0. The difference was possibly due to the presence of strawberry tree fruits (*Arbutus unedo*) and rose water in the combination, since they contain acidic compounds.

3.2.The effect of temperature on the combination

The temperature effect on the combination was studied on all of the microorganisms used in our study. There are no detailed studies concerning the effect of temperature on the action of disinfectants as well as such kind of antimicrobial substances. Various chemical and physical treatments are preferred for isolation of proteins from microorganisms, studied in different fields. On the other hand, most of these treatments have severe disadvantages. Since different criteria affect the efficiency of isolated proteins negatively, the research team of Haberl-Meglic et al. (2016) aimed to analyze the effect of temperature and microbial growth phase on the efficiency of the protein extracts. In addition, microbial viability was investigated.

The results pointed out that the temperature has a great effect on the isolation of proteins, and after the treatment, the optimum temperature is kept at 4°C. There is no microbial viability during all of the temperature assays. No effect of microbial growth phase was claimed related to the yield of protein isolation or microbial viability. Based on these experiments Haberl-Meglic et al. (2016) pointed out that the temperature is a key element for the bacterial membrane to stay in a permeabilized state, so more proteins flow out of bacteria into surrounding media. The bacterial proteins such as bacteriocins display inhibitory effect against pathogenic species like the disinfectants. The antimicrobial action of most disinfectants was reported to increase when the temperature is elevated and this is mostly related to the treatment time and temperature coefficient (Russell and Chopra, 1990), partially supporting our findings in this study.

In our experiment, the effect of different temperatures on the antimicrobial combination against the pathogenic bacteria mentioned in Materials and Method was tested by using Kirby-Bauer disc diffusion method. These pathogenic bacteria were tested at different temperatures in triplicates (4°C, 25°C, 37°C and 100°C). No significant difference among the treatments against *S.aureus* (Figure 3), *L.monocytogenes* and *K. pneumoniae* ATCC 12528 and *A.baumannii* ATCC 12365 was observed ($p>0.05$).

The combination was found to show its most strongest effect at +4 °C against *S. aureus*, the refrigeration temperature. The zone diameter of *S. aureus* was approximately 30 *mm* and the diameter was found to be 11 *mm* at 25°C, 16 *mm* at 37°C and 26 *mm* at 100°C, respectively. When temperature was increased, interestingly, a subsequent increase of inhibition was observed by raising the temperatures between 25-100° C interval. The mean value of zone diameter of *S. aureus* was recorded as 20.7 at 4°C, 13 *mm* at 25°C, 14.3 *mm* at 37°C and 17.7 *mm* at 100°C with respect to the control.

Figure 3. The effect of different temperatures on the combination against *S. aureus*.

3.3.The effect of pH on the combination

Kirby-Bauer disc diffusion method was used to observe the effect of pH on bacteria in different pHs of 2, 3, 4, 5, 6, 7, 8, 9 and 10. The adjustment of the pH of the combination was done by using 0.1 N HCl (pH=2.0) and 0.1 N NaOH (pH=13.5). 40 µL of the combination was added onto the sterile filter plates and they were incubated at 37° C for 24-48 hours. The inhibition zones were recorded in diameters and it was found to be effective against *L. monocytogenes, S.aureus, A. baumannii* ATCC 12365 and *K. pneumoniae* ATCC 12528 and a significant difference was observed in different pHs in G(+) species ($p<0.05$) (Table 5).

Figure 4. The effect of pH (2-7) against *L.monocytogenes*, tested by Kirby-Bauer disc diffusion method. The discs located in the center of the plate serve as controls.

Table 5. The mean and standard deviation results of the diameters of inhibition of zones of different pH values against different bacteria to determine the best effective dose of antimicrobial combination.

	Mean±SD			
pH values	*S.aureus*	*L.monocytogenes*	*K.pneumoniae ATCC 12528*	*A.baumannii ATCC 12365*
2	18.2500±2.4749^{b}	21.5000±2.1213^{c}	17.7500±9.5459^{a}	14.7500±3.1820^{a}
3	16.0000±1.4142^{b}	18.5000±2.1213^{c}	13.5000±3.5355^{a}	12.0000±2.8284^{a}
4	20.0000±2.8284^{c}	17.0000±2.8284^{c}	22.5000±1.4142^{a}	13.5000±3.5355^{a}
5	12.0000±1.4142^{a}	11.8000±1.4142^{b}	16.7500±1.7678^{a}	10.0000±4.2426^{a}

6	12.7500±1.0607[a]	10.750±0.3536[a]	13.5000±3.5355[a]	12.5000±2.1213[a]
7	11.0000±1.4142[a]	10.0000±0.0000[a]	12.0000±4.2426[a]	8.5000±0.7071[a]
8	16.2500±1.7678[b]	8.5000±2.1213[a]	5.0000±7.0711[a]	10.0000±0.0000[a]
9	10.5000±0.7071[b]	10.5000±0.7071[a]	5.2500±7.4246[a]	11.0000±0.0000[a]
10	10.2500±0.3536[b]	10.0000±0.0000[a]	5.2500±7.4246[a]	10.2500±0.3536[a]

*Means with the different letter indicates significant difference among the treatments against *S.aureus* and *L.monocytogenes* ($p<0.05$).

4. Conclusions

The bacteria used in our studies was inhibited by the combination as well as individual inhibition of the components was observed and they were found to be more sensitive to the combination treatments. Kirby-Bauer disc diffusion method indicated zones against the lawn cultures of the pathogens on the appropriate agars, plate count agar was used to monitor and determine the effect of the combination and it was indicated an average of 0.4-1.0 logarithmic cfu/mL decrease. Even though the rehydrated strawberry tree-rose water and silver flakes was dissolved in sterile distilled water; a good inhibitory effect was observed. Moreover, the effect of pH and temperature on the combination was found to be effective too, mostly at pH 2-6 and +4° C, respectively. In addition, each ingredient in the combination may display a synergistic characteristic to induce the inhibitory action of silver flakes, rehydrated strawberry tree fruit and rose water. It is important to conclude that although the ingredients of the combination showed inhibition against most of the pathogens used in this study; when combined, the effect was observed better than the individual ones, regarding a possible synergistic behavior.

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6. References

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