

## RESEARCH ARTICLE

# Comparison of the Effects of Silver in Nanostructured and Ultra-High Diluted Form on Growth and Volatile Compounds Produced by *Escherichia coli* and *Staphylococcus aureus*

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**Abstract: Background:** In this project, the growth and volatile metabolites profiles of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* were monitored under the influence of silver base chemical, nanoparticle and ultra-highly diluted compounds.

**Materials and Methods:** The treatments were done for 12000 life cycles using silver nanoparticles (AgNPs) as well as ultra-highly diluted Argemone nitricum (Arg-n). Volatile organic metabolites analysis was performed using gas chromatography mass spectrometry (GC-MS). The results indicated that AgNPs treatment made the bacteria resistant and adapted to growth in the nanoparticle condition. The use of ultra-highly diluted Arg-n initially increased growth but it decreased later. Also, with the continuous usage of these materials, no more bacterial growth was observed.

**Results:** The most important compounds produced by *E. coli* are Acetophenone, Octyl acetate, Styrene, 1,8-cineole, 4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane, hexadecane and 2-Undecanol. The main compounds derived from *S. aureus* are Acetophenone, 1,8-cineole, Benzaldehyde, 2-Hexan-1-ol, Tridecanol, Dimethyl Octenal and tetradecane. Acetophenone and 1,8-cineole were common and produced by both organisms.

**Conclusion:** Based on the origin of the produced volatiles, main volatiles percentage of untreated sample is hydrocarbon (>50%), while bacteria treatments convert the ratio in to aldehydes, ketones and alcohols in the case of AgNPs, (>80%) and aldehydes, ketones and terpenes in the case of Arg-n (>70%).

**Keywords:** Ultra-high diluted compound, silver nanoparticle, *Staphylococcus aureus*, *Escherichia coli*, volatile metabolite, gas chromatography.

## 1. INTRODUCTION

In the eighteenth century, Samuel Hahnemann, a German physician, discovered homeopathy, an alternative and complementary system of medicine<sup>1</sup>. This method uses ultra-highly diluted compound routinely from the nano ( $10^{-9}$ ) or yocto ( $10^{-24}$ ) dilution to  $10^{-500}$  dilution [1, 2]. Since these compound are available in ultra-high dilutions, and according to Avogadro's hypothesis, these compound are nothing more than a "placebo" [1, 3], but they influence on humans [3e], animals and plants [3f, g]. The two main questions related to this are: (a) how is medicinal property transferred into vehicle substance by serial dilutions? and (b) what is the physiological mechanisms of these ultra-highly diluted tiny

globous compound? Answering these questions looks easy at the beginning, but because the answer is related to different domains of science, including physics, physiology/molecular biology and psychology/medical science in addition to biochemistry and also biophysics, this question remains unanswered and complicated [4]. Thus, the study of these ultra-highly diluted compound has now become a new and upcoming interdisciplinary field [5]. It is true that the action mechanism is still unclear, but this is the essence of scientific discovery and instead of the Denning method as a result of the inadequate information, there is a need for investigations [2a, c, 3a]. The simple questions are as follows: What would be the outcome if inculcation creatures such as animals, plants and microorganisms are unable to respond to these remedies? Is there a possibility of response for simple single-celled organisms? One of the most important and common causes of diseases are bacteria [6]. It has been discovered that silver and its related compounds are effective anti-microbial agents [7].

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Silver nanoparticles (AgNPs) as a new form of silver represent a novel generation [8] of anti-microbial [9], anti-fungal and anti-viral applications against a broad range of micro-organisms [10]. These days, in our daily life, we are faced with a wide range of products [9] from clothing and packing to herbicides and pesticides, containing AgNPs [11], and their toxic effects and environmental contaminations cannot be ignored [10b-d]. In addition, the use of these compounds leads to bacterial mutation into resistant species [10c]. Thus, there is a need to increase the dose used as well as the antibacterial features. So far, this method has no healing effect and is however, doomed to exclusion.

Silver in its various forms from the past and today in the nanoscale form has been used as antibacterial [12]. The mechanism and the mode of interaction of different silver structures against bacteria are different and require more exploration [13]. The volatile compounds produced by bacteria indicate their metabolism variations [14] in conditions of normal growth, resistance [15], apoptosis or even necrosis [16]. According to what has been described, how can the composition of different silver compounds, chemical molecules, nano and ultra-highly diluted compound act very differently? One causes complete growth ban and death, the other with a high toxicity, even to the host, results in mutation into a resistant strain [17], and third, without showing toxic effects and based on the host characteristics, can lead to the removal of bacterial infections. This question is very important: what are the action mechanisms of these compounds in the process of growth, vitality and metabolism of bacteria? The purpose of this project was to study the growth and volatile metabolite profiles of the effects of a silver base chemical, nanoparticle and ultra-highly diluted compound, on Gram positive and negative model bacteria, *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

## 2. MATERIALS AND METHODS

### 2.1. Model Organisms, Material and Treatment Conditions

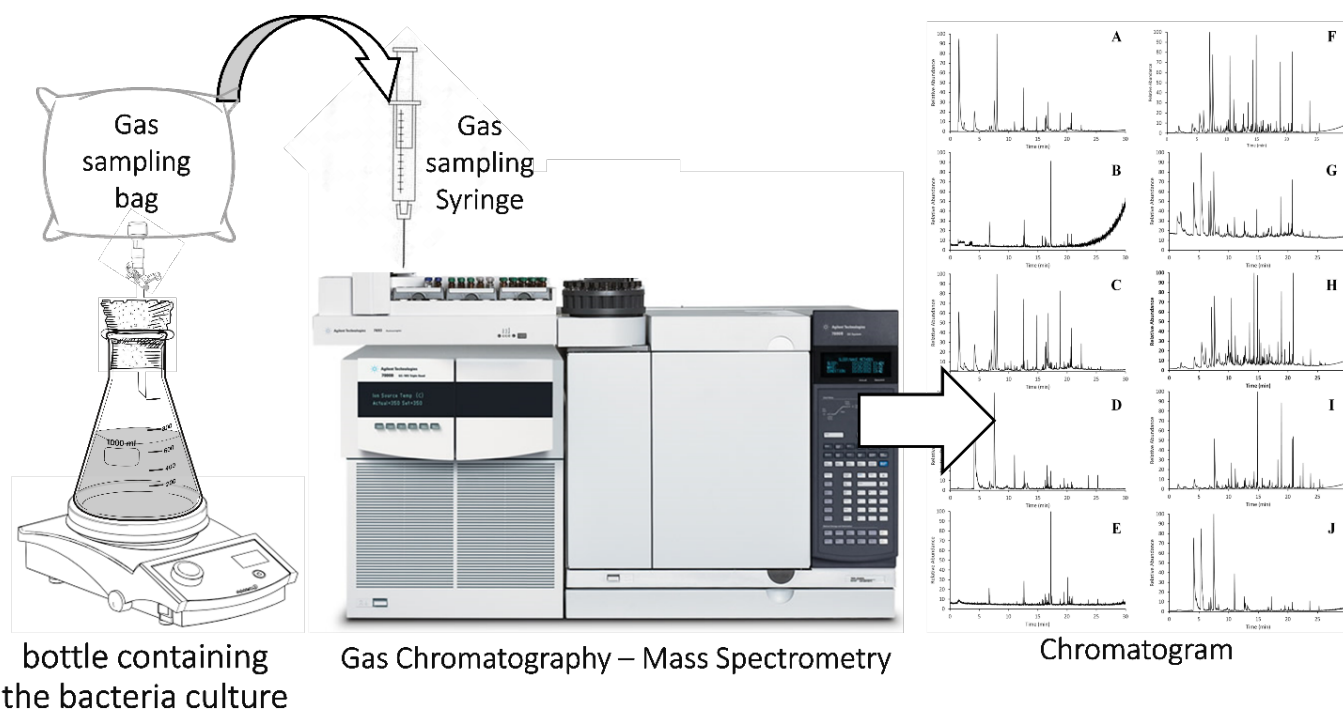
The bacterial strains used in this study are *E. coli* (ATCC 25922) as Gram-negative and *S. aureus* (ATCC 25923) as Gram-positive model organisms. These model organisms were obtained from the Microbiology Laboratory of Medicinal Plants and Drugs Research Institute, Shahid Beheshti University. Monocultures of all strains were cultured for 24 h in nutrient agar, and then sub-cultured aerobically at 37°C in 30 ml of Mueller Hinton Broth (MHB) in 100 ml sterilized glass bottles.

The treatments were organized in order to study the AgNO<sub>3</sub> (Sigma-Aldrich Co., Germany) biocidal activity and its comparison with the nano form (Silver nanoparticle: AgNPs) and ultra-highly diluted compound (Argentum nitricum, Arg-n). The homemade AgNPs were prepared and characterized according to the previously reported method [17]. Briefly, colloidal AgNPs with spherical morphology and distinct diffraction peaks of the crystalline plans of cubic AgNPs including (111), (200), (220) and (311), have a maximum absorbance ( $\lambda_{max}$ ) at 428 nm. Their size was  $19.20 \pm 0.50$  nm (X99) with the homogeneity of their size being within the range of 0.1–1000 nm. The Arg-n

globules (DHU Co., Germany), in the potency of C30 (30 times dilution in the ratio of 1:99) was used. These two forms of Ag were compared with AgNO<sub>3</sub>, (Sigma-Aldrich Co., Germany), with the bactericidal concentration of 0.0001 M. In each case (AgNO<sub>3</sub>, AgNPs and Arg-n), the growth curve was registered and the life cycle of both the model bacteria was found to be approximately 5 h. The treatments were performed for 12000 life cycle (~210 days). In every 500 life cycle (every ~10 days), once at the middle of the log exponential phase (after 2.5 h), the bacteria were treated (using AgNO<sub>3</sub>, AgNPs or Arg-n) in the MHB media (500 mL media in a 2 L volumetric flask), incubated for 18 h in the growth indicator, optical density at OD600, and cell viability (using an optical microscope) were monitored. Thereafter, 1 mL of the obtained suspension was transferred to MHB and passaged (sub-culturing) for the next 10 days. In this study, 500  $\mu$ l of sampling was done every day and the growth curve was plotted based on daily samples (Fig. 1). In this article, and based on our previous researches on AgNPs interaction with bacteria [7, 10b, c], the homemade colloidal aqueous AgNPs in the concentration of  $100 \mu\text{g}/\text{mL}$  was used for the treatment of the model bacteria: *S. aureus* and *E. coli*. The third form of silver that was studied is the ultra-highly diluted C30 (30 times dilution in the ratio of 1:99), and potent compound. The globules were dissolved in double distilled water and shaken vigorously for 1 min, prior to application on the bacteria. The growth of each bacterium in MHB without treating material was used as a blank condition. Each examination was performed in three replications. After the daily passage, volatile organic compounds were extracted from 400 mL of bacterial liquid medium and kept in a suitable sampling bag (Tedlar bag, TI00.5 L, Techinstro Co., India) at -80°C. To increase the possibility of VOC production, bottles containing cultured microorganism were shaken at 150 rpm during the incubation time, Scheme 1. To provide conditions that increase the rate of VOC absorption, 2 ml of NaCl 36% was added to each culture. Then, a sampling bag was suspended from the top of the bottle containing the culture and placed on a magnetic stirrer hotplate at 70°C for 30 min. According to the growth curve trend, samples were selected and analyzed using GC-MS, Scheme 1.

#### 2.1.1. Gas Chromatography Mass Spectrometry Analysis of Volatile Organic Compounds

Volatile Organic Compounds (VOCs) on the growth condition headspace produced by each microorganism were chosen and analyzed based on growth curve trend. Eventually, each VOC was represented as a chromatogram peak in the monitor connected to the GC-MS. To study the bacterial VOCs, a Thermo-Finnigan Trace GC-MS system (Thermo Quest-Finnigan Co., USA) equipped with a DB-5 column (60 m length, 0.25 mm inner diameter and 0.25  $\mu$ m film thickness) with helium carrier gas at a flow rate of 1.1 ml/min was used. The starting temperature was 50°C, increasing at a rate of 10°C/min up to 250°C. The GC-MS was set in splitless mode and a quadrupole ion trap with ionization energy of 70 eV was used in the filament. VOCs were identified using the National Institute of Standards and Technology (NIST) reference library. To analyze the GC-



**Scheme 1.** The process and equipment used for VOCs extraction and determination.

MS data, Xcalibur 3.0 with Foundation 3.0 SP2 software (ThermoFisher Scientific., USA) was used, and the Kovats Retention Index (RI) was calculated for each chromatographic peak. The Xcalibur raw files for these pathogens are available at <https://doi.org/10.6084/m9.figshare.5178004.v132>. When calculating the RI, a series of standards were used: n-alkanes were injected into the GC-MS a day before starting the experiments, using the same temperature profile used for the analysis of VOCs. The NIST17 Mass Spectral Library (NIST7/2017/EPA/NIH) was used to identify each compound according to its RI.

### 3. RESULTS AND DISCUSSION

#### 3.1. AgNO<sub>3</sub>, AgNPs and Arg-n Treatment and Growth

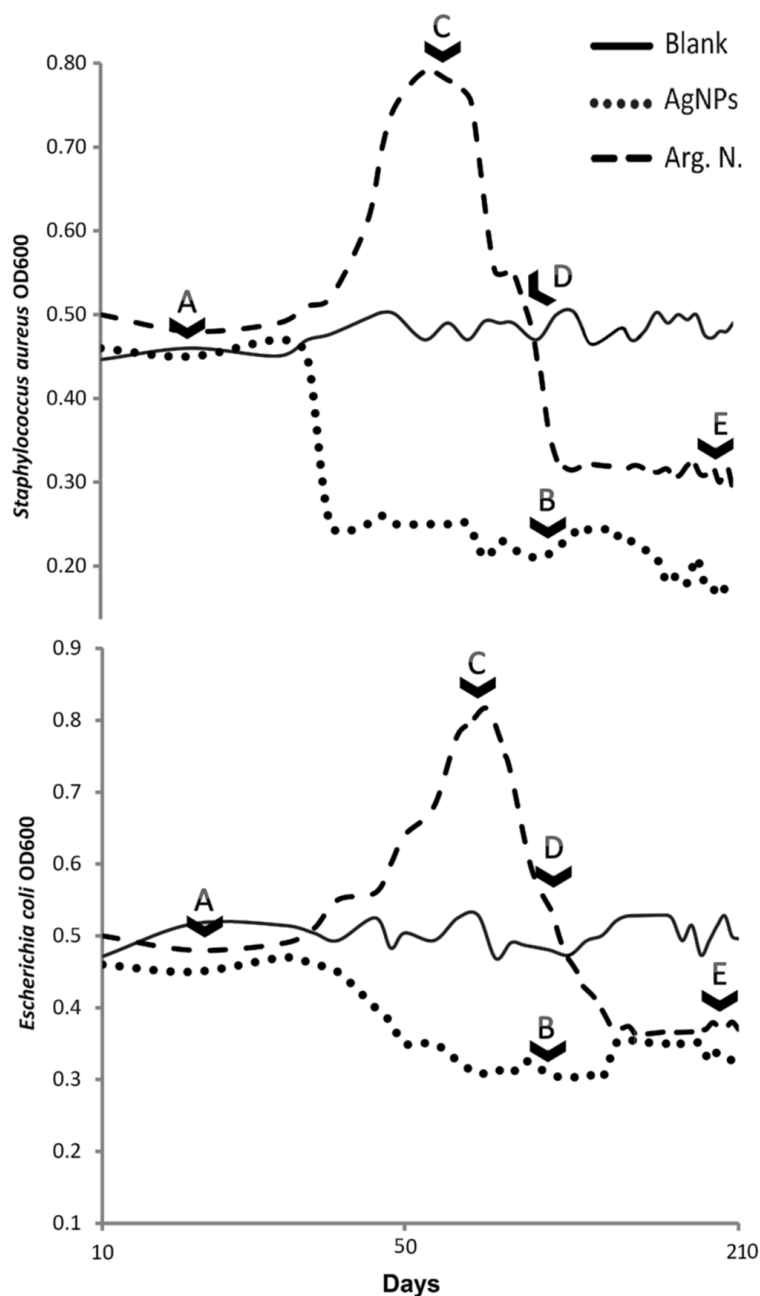
The VOCs produced by *S. aureus* and *E. coli* were assessed under three different silver treatments (AgNO<sub>3</sub>: 0.001 M, AgNPs: 100 µg/mL, and Arg-n: C30). AgNO<sub>3</sub> was used as an antibacterial a long time ago [18]. It is a very powerful compound and has a very fast and intense ability to remove bacteria. Further studies have shown that this silver form is highly toxic, dangerous and mutagenic. Therefore, its use as an antibacterial in products and sterilization methods has become obsolete [12-19]. The bacteria treated with AgNO<sub>3</sub>: 0.001 M, which is a biocidal concentration, did not show any growth. The growth possibility is checked for 20 days, but no cultivation was observed. As a result, this sample with the highest bactericidal effect was excluded from the study.

Previously, after improving the high toxicity of AgNO<sub>3</sub>, the usage of silver salts for antibacterial activity decreased dramatically, until its nanoparticles were invented [20]. With the beginning of nanoparticle applications and the passage of time, the unwanted and toxic effects of these compounds were identified [7-10b]. As reported, the toxicity of both

bare and coated silver nanoparticles increased as time increased, yet the nanoparticles attenuated the general toxicity by metal ion release [17-21]. Finally, environmental risk assessments of AgNPs should include chronic toxicity tests for aquatic organisms as well as the direct and indirect effects of AgNPs, resulting from the release of Ag<sup>+</sup> into the environment [22]. However, given the less destructive effects of AgNPs compared to silver salts, it is continuously used as an antibacterial [23]. In the case of long-term applications of AgNPs, there is a hypothesis of bacterial mutation into resistance types [13]. As shown in Fig. (1), after the AgNPs treatment, the optical density decreased drastically. In the case of *S. aureus*, no growth was observed during the subsequent passages, but a small amount of growth was observed in the case of *E. coli* after the 14<sup>th</sup> passage. Using the same dosage in the remaining seven passages, the treatment did not influence the growth of *E. coli*. From these results, it can be hypothesized that, the AgNPs with the mentioned characteristics and concentration, after 140 days treatment on *E. coli*, can result in resistance of the bacteria. This prediction is not discussed in this article, but there is a possibility for research in this regard.

The third form of silver that was studied is the ultra-highly diluted C30 (30 times dilution in the ratio of 1:99), and potent compound. The results are remarkable and interesting. Initially and after 1 treatment (within 10 days), there was an increase in bacterial growth. This growth increment was maintained for *E. coli* for up to 4 treatment steps (40 days) and for *S. aureus* for up to 5 treatment steps (50 days) and then reduced. After drastic reductions, both bacteria had no more growth in the remaining 7 treatment steps (~70 days).

As shown in Fig. (1), five samples in each bacterial media (overall 10 samples) were chosen for volatile organic metabolite analysis using GC-MS. One sample is the sample of the passage but was not treated (A). The second sample

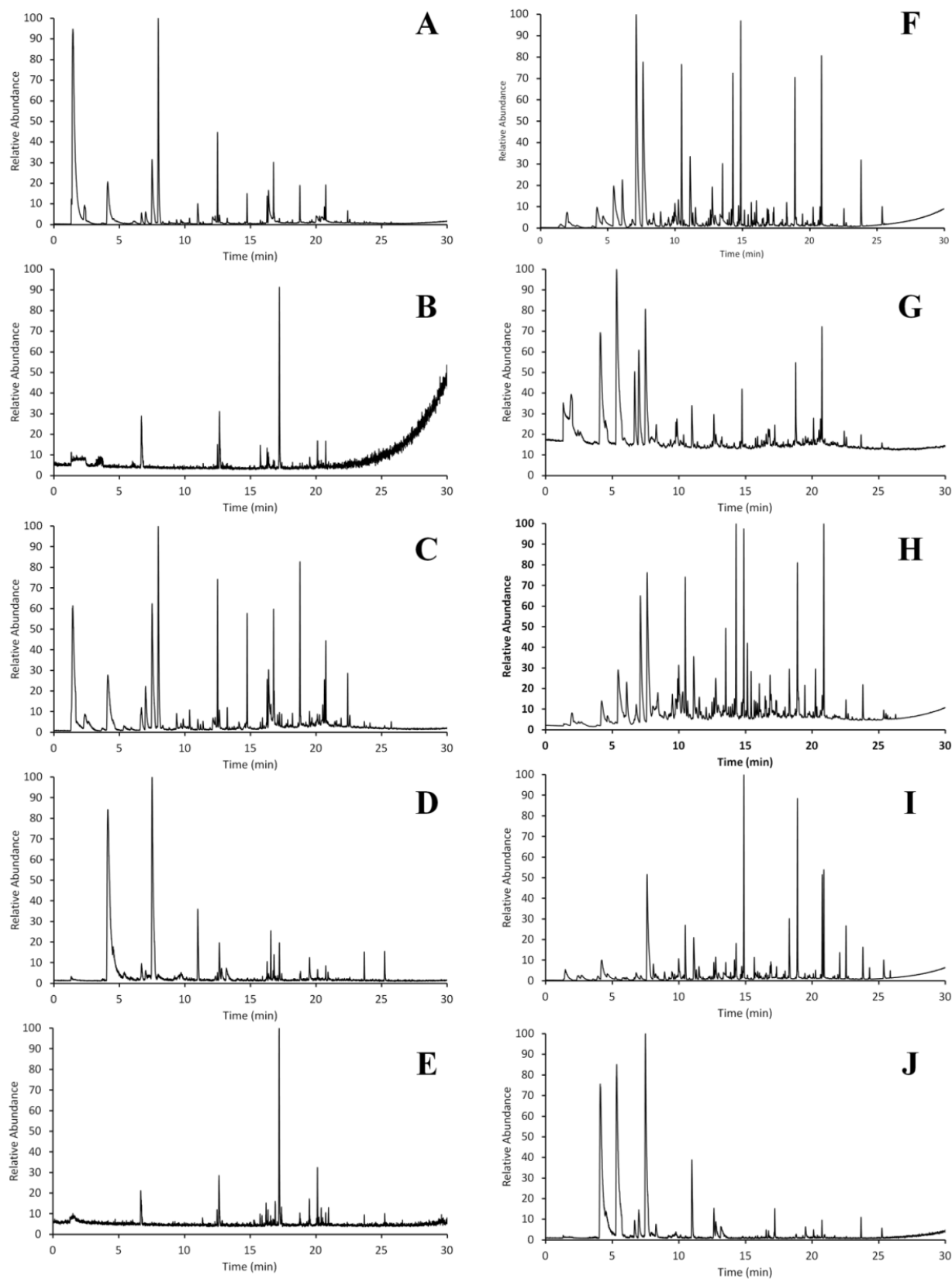


**Fig (1).** Growth study of *S. aureus* and *E. coli* over 1000 life cycle (~ 210 days) of treatments at OD 600 nm. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

refers to the treated bacterium with AgNPs, which showed a growth inhibition (B). The next specification is related to the maximum growth conditions of the bacterium treated with Arg-n (C). Two samples were taken from the same treatment in the middle of the decrease in growth (D), and the end stages of bacterial growth (E). The GC-MS analysis results of the aforementioned treatments are shown in Fig. (2) and the details of the compounds identified and the frequency of each are shown in Tables 1 and 2.

As shown in Fig. (2), chromatograms A and F are related to the escape of two bacteria, *E. coli* and *S. aureus*, respectively. The details of the material identification are shown in

Tables 1 and 2. According to these tables, the most important (more than 5%) compounds with 99.17% identification in *E. coli* are Acetophenone (24.1%), Octyl acetate (9.8%), Styrene (5.92%), 1,8-cineole (5.8%), 4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane (5.35%), hexadecane (5.26%) and 2-Undecanol (5.05%). The main compound (more than 5%) of 99.7% total identification derived from *S. aureus* are Acetophenone (13.97%), 1,8-cineole (10.14%), Benzaldehyde (7.1%), 2-Hexan-1-ol (5.75%), Tridecanol (5.05%), Dimethyl Octenal (5%) and tetradecane (4.59%). Acetophenone and 1,8-cineole were common and produced by both organisms. From Fig. (3), it can be concluded that these VOCs can be more important in organisms producing greater quantities.



**Fig (2).** Volatile compound gas chromatography- mass spectrometry of *E. coli* (left) and *S. aureus* (right) over the mentioned treatment on fig.1, no treatment (A/F), AgNPstreatment (B/G), maximum growth under the Arg-n treatment (C/H), middle growth decrement under the Arg-n treatment (D/I), and the end stages of bacterial growth under the Arg-n treatment (E/J). the analysis perform on GC-Ms by DB-5 column (60 m, 0.25 mm ID, and 0.25  $\mu$ m film thickness) with helium carrier gas at a flow rate of 1.1 mL/min, the starting temperature was 50  $^{\circ}$ C, increasing at a rate of 10 $^{\circ}$ C/min up to 250 $^{\circ}$ C in 30 min. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

Table 1. Volatile identified metabolite from *S. aureus*.

No	RI	Compound	Percentage				
			A	B	C	D	E
1	843.13	Styrene	3.47	16.48	6.2	25.1	7.96
2	1428.64	Myrcene	-	-	-	1.94	-
3	1423.64	Docosane	-	-	-	1.41	-
4	1301.64	Indole	2.58	0.11	-	-	-
5	1568.02	Nonadecane	0.98	0.43	-	1.02	2.98
6	1329.26	Longifolene	-	0.17	-	-	0.42
7	1598.45	(E)-2-hexyl ester- Butanoic acid	-	-	-	4.79	-
8	968.13	Benzaldehyde	7.1	29.39	0.4	1.26	-
9	1036.04	1,3-Butadiene	1.26	-	1.14	1.31	0.8
10	1016.04	1,8-cineole	10.14	9.08	1.55	9.51	-
11	1097.04	Acetophenone	13.97	14.14	17.95	20.23	24.44
12	1158.97	3-Methyl-1,5-heptadiene	0.64	2.58	0.24	0.7	-
13	1659.02	Cedran-1,8-diol	-	0.62	-	0.95	-
14	1741.58	2-(phenylmethylene)-Octanal	-	-	1.73	-	0.26
15	1436.64	Tetradecane	4.59	1.5	7.52	-	1.23
16	1823.64	Octacosane	-	-	-	-	0.28
17	1549.45	Epicedrol	-	-	8.16	0.34	0.47
18	1396.26	1,9-Decadiene	0.56	0.15	1.26	0.69	-
19	1208.98	2-Undecanol	1.13	0.15	3.58	-	0.61
20	1225.97	1-Decyne	0.38	2.39	3.47	3.42	0.79
21	1514.64	Cadinene	0.22	0.13	-	0.21	0.47
22	1484.26	1-(1,5-dieethyl-4-hexyl)-4-methyl-Benzene	-	0.26	-	0.27	-
23	1405.45	2,5-(1,1-dimethylethyl)-Phenol	1.1	-	0.52	-	-
24	871.13	Limonene	-	-	-	2.49	1.94
25	1521.97	trans-Caryophyllene	-	-	0.65	-	1.09
26	1144.98	2-Undecanone	0.13	0.41	0.27	0.35	-
27	1539.64	3-methyl-Pentadecane	-	0.29	-	0.15	0.5
28	1517.45	Dodecenol	0.25	-	-	-	0.91
29	1583.64	Pentadecane	-	-	-	0.25	0.87
30	1344.26	o-isopropylanisol	2.16	0.22	1	-	-
31	1569.02	Tetradecanol	-	-	0.86	-	-
32	1350.64	2-Decanone	0.2	1.6	0.74	-	-
33	1550.45	Benzophenone	1.85	-	1.38	-	0.2
34	1632.02	Cedrol	0.19	0.94	0.64	-	-
35	1477.26	Octyl acetate	0.44	0.21	0.24	-	-

Table 1. Contd...

No	RI	Compound	Percentage				
			A	B	C	D	E
36	1372.26	Dimethylethyl Cyclohexanol	1.42	0.13	0.63	-	0.41
37	1162.98	Methone	1.25	-	1.77	-	4.96
38	1621.58	Tridecanol	5.05	0.53	5.81	-	0.58
39	1500.26	5,5-Dodecadinyl-1, 12-diol	0.22	-	0.53	0.67	-
40	1434.26	2,3-Hexandione	0.9	0.51	-	-	-
41	1674.02	5,8-Diethyl-6-dodecanol	-	-	-	0.45	0.63
42	1259.97	Dodecanal	0.27	0.1	0.56	-	-
43	1634.02	Eicosane	-	-	-	1.35	-
44	888.13	Carbamic acid	0.64	-	0.48	1.61	1.82
45	1449.45	Caryolan-8-ol	0.7	0.17	-	-	-
46	1598.45	Hexadecane	3.78	2.96	7.22	-	-
47	1454.45	Sesquiesabinene	-	0.15	0.43	0.11	-
48	1358.26	4-Butoxy-1-Butene	0.45	0.14	-	-	-
49	1378.64	2-Hexan-1-ol	5.75	-	1.53	-	-
50	1015.04	$\alpha$ -terphenyl acetate	-	-	-	-	6.46
51	1688.02	$\beta$ -Santalol	-	-	0.39	-	-
52	1699.02	7-methoxy-6-ethoxy-2,2-dimethyl-2-chromene	0.4	0.39	0.21	-	-
53	1297.97	butyraldehyde	0.49	0.18	0.35	0.26	-
54	1697.02	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane	3.9	-	0.25	0.83	-
55	1608.58	2-Piperidinone	-	3.59	-	0.56	-
56	1230.97	Indolizine	-	-	0.9	0.38	2.98
57	1582.45	10-Methylnonadecane	-	0.14	-	0.17	-
58	1337.64	Aromadendrene	0.24	-	0.38	-	-
59	1458.98	(z)-2-Octene-1-ol	-	-	-	-	3.58
60	1659.02	Dibutyl phthalate	0.29	-	-	0.13	0.6
61	1204.97	1-Naphthalenol	3.49	0.27	1.33	0.32	0.74
62	1648.02	Selinene	1.76	0.51	-	-	-
63	1134.98	Cyclohexane propanol	0.21	0.44	0.16	0.99	3.98
64	1465.97	1,5-Decadiene	-	-	-	-	0.29
65	1246.97	2-methyl Naphtalene	1.03	-	1.42	0.15	1.02
66	1788.58	Phthalic acid, butyl ester	0.51	0.17	-	0.23	3.61
67	1190.97	2-Naphthalenol	-	-	-	3.51	2.03
68	943.04	Decane	2.84	5.85	0.27	-	-
69	1408.26	2-ethenyl-6-methyl-Pyrazine	0.46	0.35	-	0.55	-
70	1141.97	Dimethyl Octenal	5	0.53	-	-	-
71	1139.04	1-Undecenone	-	-	-	-	8.82

Table 1. Contd...

No	RI	Compound	Percentage				
			A	B	C	D	E
72	1052.98	2,3-Pentandione	0.64	0.98	0.68	1.33	2.39
73	1369.97	2-methyy-5-(1-methylethyl)-Phenol	1.6	-	-	-	-
74	1498.26	Zingiberene	-	0.45	0.35	0.9	1.8
75	1444.97	6-Methyl-5-hepten-2-one	0.29	0.1	-	0.12	0.44
76	1507.45	5-methyl-Pentadecane	-	0.3	0.35	0.44	-
77	1855.26	1,2-Benzenedicarboxylic acid	-	-	-	-	1.61
78	1123.97	isocyanomethyl Benzene	1.63	-	0.23	0.72	-
79	1420.26	Nerylacetone	0.36	-	-	-	-
80	1141.97	Dodecane	-	-	2.85	-	-
81	1190.98	2-Heptanone	0.79	0.89	0.24	0.21	-
82	1227.64	1-(1-hexyl)-cyclohexanol	-	0.59	0.62	-	-
83	1721.04	Decyl-Cyclohexane	-	-	5.42	-	0.69

Table 2. Volatile identified metabolite from *E. coli*.

No	RI	Compound	Percentage				
			A	B	C	D	E
1	845.13	Styrene	5.92	0.2	9.06	22	3.4
2	941.04	Benzaldehyde	0.62	0.1	1.88	1.34	0.99
3	1015.04	Carbamic acid	-	1.2	-	-	-
4	1016.04	1,8-cineole	5.8	-	1.61	6.56	0.86
5	1039.98	cyclohexane propanol	0.94	-	-	0.58	0.44
6	1053.98	2,3-Pentandione	0.28	0.34	12.6	0.18	0.55
7	1097.04	Acetophenone	24.1	15	2.14	20	0.71
8	1121.97	isocyanomethyl Benzene	0.3	-	-	-	6.62
9	1139.04	1-Decene	0.2	0.64	-	-	0.87
10	1141.97	Dimethyl Octenal	1.79	-	-	0.4	3.28
11	1144.98	2-Undecanone	0.18	0.81	1.75	4.82	0.55
12	1157.98	Methone	2.8	-	0.44	-	1.09
13	1189.98	2-Heptanone	0.38	-	1.73	0.2	0.28
14	1204.97	3-Methyl-1,5-heptadiene	4.19	0.09	-	-	1.98
15	1208.98	2-Undecanol	5.05	-	-	-	1.8
16	1226.97	Indolizine	0.83	-	1.55	0.15	1.66
17	1227.64	2-methyy-5-(1-methylethyl)-Phenol	2.85	-	-	-	24.8
18	1227.64	1-(1-hexyl)-cyclohexanol	-	16	-	-	-
19	1237.64	2-octyl-1-ol	-	-	1.55	-	-

Table 2. Contd...

No	RI	Compound	Percentage				
			A	B	C	D	E
20	1247.97	2,4,5-trimethyl Benzeneamine	0.87	-	-	-	2.34
21	1283.64	Butyraldehyde	0.22	-	6.51	-	2.52
22	1300.64	Indole	0.61	24	-	0.78	1.19
23	1317.26	Tetradecane	-	-	1.37	-	-
24	1320.97	o-Isopropylanisol	0.21	0.54	-	0.84	3.39
25	1321.64	(z)-2-Octene-1-ol	0.39	-	0.19	-	0.59
26	1321.64	Aromadendrene	-	-	0.19	-	-
27	1337.64	trans-Caryophyllene	0.39	-	-	-	0.56
28	1341.97	2-Decanone	0.51	0.4	6.91	1.84	2.32
29	1344.26	4-Butoxy-1-Butene	0.78	-	0.28	-	1.15
30	1355.64	2-Hexan-1-ol	0.3	-	1.24	-	1.82
31	1357.97	Naphthalenol	0.27	8	1.55	2.87	3
32	1372.64	Dimethylethyl Cyclohexanol	0.18	0.56	-	-	0.97
33	1396.26	1,9-Decadiene	0.19	-	1.23	0.56	-
34	1406.26	2-Nonanone	-	-	7.55	0.84	-
35	1413.45	2-ethenyl-6-methyl-Pyrazine	2.24	0.95	0.2	0.22	-
36	1420.64	Longifolene	0.8	0.69	0.2	-	1.18
37	1423.64	(z)-4-Decan-1-ol	-	-	6.51	1.25	-
38	1434.26	2,3-Hexandione	1.67	0.6	0.34	0.73	-
39	1441.45	Caryolan-8-ol	0.52	0.53	5.23	-	-
40	1441.45	2-methyl-1-Propanol	-	-	1.23	0.45	-
41	1441.45	1,5-Decadiene	-	0.79	0.34	-	-
42	1454.45	Sesquiesabinene	-	0.1	-	4.27	-
43	1464.26	Octyl acetate	9.8	0.9	-	-	-
44	1484.26	5.5-Dodecadinyl-1, 12-diol	0.18	0.77	-	0.14	-
45	1498.26	1-(1,5-dieethyl-4-hexyl)-4-methyl-Benzene	1.42	-	-	-	-
46	1498.26	Zingiberene	-	2.16	-	-	-
47	1506.45	Cadinene	0.25	-	2.71	-	-
48	1512.02	Hexadecane	5.26	0.18	-	-	-
49	1514.26	2,5-(1,1-dimethylethyl)-Phenol	1.08	0.58	0.25	-	-
50	1517.45	Tetradecanal	0.3	-	-	-	-
51	1540.02	3-methyl-Pentadecane	0.2	0.2	-	0.1	-
52	1553.45	Epicedrol	0.31	-	-	-	-
53	1564.45	Tetradecanol	0.11	0.48	0.29	-	-
54	1574.45	Pentadecane	0.19	-	-	1.21	2.46

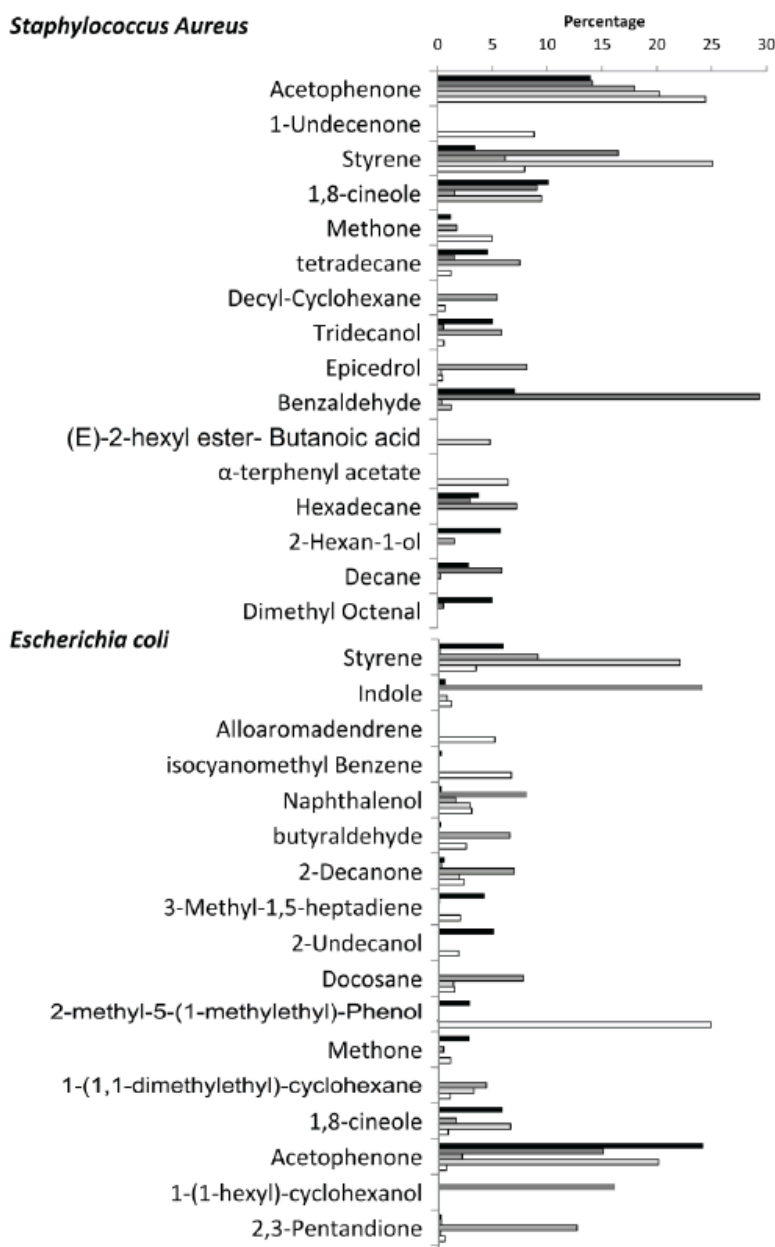
Table 2. Contd...

No	RI	Compound	Percentage				
			A	B	C	D	E
55	1583.45	6-Methyl-5-hepten-2-one	-	0.28	-	0.97	-
56	1583.45	10-Methylnonadecane	-	0.3	-	2.17	-
57	1596.45	(E)-2-hexyl ester- Butanoic acid	0.18	0.25	3.05	-	-
58	1598.45	Heptadecane	-	-	-	2.53	-
59	1604.02	2,6,10-trimethyl-Pentadecane	0.35	5.84	-	-	-
60	1608.58	1-Dodecane	-	-	-	5.93	-
61	1609.58	1-(1,1-dimethylethyl)-cyclohexane	-	-	4.32	3.19	1.05
62	1610.58	Dodecenol	0.41	5.13	-	-	-
63	1621.58	4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane	5.35	-	0.04	-	-
64	1632.02	Cedrol	0.21	0.32	0.88	1.61	-
65	1634.02	Alloaromadendrene	-	-	-	-	5.1
66	1648.02	Benzophenone	1.84	0.57	-	1.56	-
67	1659.02	5,8-Diethyl-6-dodecanol	0.26	-	0.76	-	-
68	1664.02	Tridecanol	-	-	-	0.48	0.48
69	1687.02	Docosane	-	-	7.74	1.33	1.43
70	1687.02	Cedran-1,8-diol	0.73	-	-	-	-
71	1687.02	2-Piperidinone	-	2.49	-	-	-
72	1690.58	$\beta$ -Santalol	-	-	0.08	-	-
73	1717.05	Decyl-Cyclohexane	0.44	-	0.51	0.4	0.49
74	1734.05	7-methoxy-6-ethoxy-2,2-dimethyl-2-chromene	0.76	-	0.18	-	0.19
75	1734.05	2-(phenylmethylene)-Octanal	-	-	0.04	-	-
76	1775.58	6-Dodecane	-	-	-	3.55	-
77	1775.58	Nonadecane	-	-	0.24	0.61	1.32
78	1832.05	Octacosane	-	-	-	0.35	0.52
79	1832.05	5-methyl-Pentadecane	-	-	-	0.14	-
80	1845.05	Selinene	2	-	3.05	-	0.15
81	1855.29	Dibutyl phthalate	0.4	-	-	-	3.11
82	1930.29	Phthalic acid, butyl ester	0.76	-	0.14	-	3.19

As a result, in the treatment of *S. aureus* with AgNPs, some compounds including Benzaldehyde, Styrene, Acetophenone and Decane significantly increased, while 1,8-cineole decreased. Also, 2-Piperidinone, Cedran-1,8-diol, 1-(1-hexyl)-cyclohexanol, Zingiberene, 5-methyl-Pentadecane, 3-methyl-Pentadecane, 1-(1,5-dieethyl-4-hexyl)-4-methyl-Benzene, Longifolene, Sesquiesabinene and 10-Methylnonadecane were identified as not produced in the untreated *S. aureus*. In the case of *E. coli*, while Acetophenone, Octyl acetate, Styrene and hexadecane decreased, thirty compounds in which some of the important ones are 1,8-cineole, 4-t-butyl-2-(1-methyl-2-nitroethyl)cyclohexane and 2-Unde-

canol, were not produced any more. In addition, the AgNPs treatment on *E. coli* caused the production of 1-(1-hexyl)-cyclohexanol, 2-Piperidinone, Zingiberene, Carbamic acid, 1,5-Decadiene, 10-Methylnonadecane, 6-Methyl-5-hepten-2-one and Sesquiesabinene.

Considering what Arg-n C30 has on the volatile metabolites, some compounds were produced under the Arg-n treatment including: Indolizine, Zingiberene and Epicedrol in the case of *S. aureus* and 1-(1,1-dimethylethyl)-cyclohexane, Docosane and Nonadecane in the case of *E. coli*. On the other hand, some compounds were removed: mainly Indole, Nonadecane and Selinene in the case of



**Fig (3).** *S. aureus* and *E. coli* main volatile compound variation under the treatment conditions mentioned in Fig. (1). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

*S. aureus* and 3-Methyl-1,5-heptadiene, Octyl acetate, Hexadecane and Benzophenone in the case of *E. coli* (Tables 1 and 2). After treatment of *S. aureus* with Arg-n, four compounds: 1,8-Cineole, Benzaldehyde, 2-Hexan-1-ol and Dimethyl Octenal were severely reduced or eliminated, but other compounds including Acetophenone, Epicedrol, Tetradecane, Hexadecane, Styrene, Tridecanol and Decyl-Cyclohexane were produced or significantly increased. The same was observed in the case of *E. coli*. In this case, 2-Undecanol, Acetophenone, Octyl acetate, Hexadecane and 4-t-Butyl-2-(1-methyl-2-nitroethyl) cyclohexane decreased or not produced, while 2,3-Pentandione, Styrene, Docosane, 2-Nonanone, 2-Decanone, (z)-4-Decan-1-ol, Butyraldehyde and Caryolan-8-ol were produced or increased in quantity (Tables 1 and 2; Fig. 3).

With the continuation of Arg-n bacterial treatment, the variety and amount of produced compounds changed (Tables 1 and 2). It is better to examine the process of change of some of the most important compounds. Acetophenone, the simplest aromatic ketone with the ability to reduce and adjust the intensity of the odor and an absorbent substance, is one of the most important compound produced in both bacteria. The *S. aureus* treatment with Arg-n increased its amount. With increase in the amount of this compound, reduction in the unpleasant smell can be expected. The production pattern of Acetophenone in the AgNPs or Arg-N treatment of *E. coli* shows decrease in the production amount, especially in Arg-N treatment, (Fig. 1, lower-step C). Under Arg-n treatment, the production of Acetophenone initially increased and with further treatment, it was reduced again (Fig. 1, steps C, D and E and Table 2).

The next compound that can be considered more closely is 1,8-Cineol, a cyclic ether and a monoterpene with strong and cold odor. The amount of 1,8-Cineol decreased in both bacteria under AgNPs and Arg-n treatments. Continuation of the Arg-n treatment (Fig. 1) causes its increase (step D), followed by a marked decrease (step E). The next noticeable compound is Styrene, a derivative of benzene that has a sweet but less pleasant smell. The treatment of *S. aureus* with AgNPs and Arg-n causes increase and decrease in Styrene, respectively (Table 1). In the case of *E. coli*, both AgNPs and Arg-n treatments caused the decrease (Table 2).

Benzaldehydes, consisting of a benzene ring with a formyl substituent and having a strong bitter almond odor, is one of the compounds produced by *S. aureus*, and it increased under AgNPs treatment but decreased under Arg-n treatment (Table 1). This compound is not the main product of *E. coli* (Table 2). Indole, an aromatic heterocyclic organic compound produced by a variety of bacteria under environmental stress and resistance to death, is the main compound produced by *E. coli* under AgNPs treatment. This compound has a nauseous smell. According to previous studies, the major volatile produced by an untreated bacterium has a simple and hydrocarbon base metabolic pathway.

## CONCLUSION

By showing that silver nitrate is highly bactericidal, this study suggests that ultra-high diluted compounds (*Argentum nitricum* 30C) affects bacteria *E. coli* and *S. aureus in vitro*. This is shown in two ways. First, the ultra-high diluted compounds increase bacterial growth for some days and then decrease it. Secondly, Volatile Organic Compound (VOC) study of bacteria shows variation in volatile metabolite profile when treated with the ultra-high diluted compounds. The same study has also been carried out with AgNPs. It was found that AgNPs stop the bacterial growth, but after some days, bacterium *E. coli* starts growing again and it seems that they become resistant to used AgNPs. However it shows a small growth decrement in 210 days and may need more days of study for improving this hypothesis. This, however, does not happen with the ultra-high diluted compounds. Chemical/physical reports on ultra-highly diluted compounds ultra-high diluted compounds indicate that the dilution process together with succession cause changes in the structure and arrangement of water molecules [5, 24, 25]. The precise mechanism(s) of highly diluted and patented remedies are still unknown [5, 24-26]. This project investigated the effects of raw materials, nanoparticles as well as the ultra-highly diluted state of silver nitrate on two model bacteria: *S. aureus* and *E. coli*. Also, the possible effects on their growth pattern as well as the metabolites produced, were evaluated. The results indicate that the pattern of bacterial growth varied in the presence of each of the mentioned materials. The silver nitrate in the first treatments resulted in death, stopped re-growth, and was eliminated from the studies. Firstly, the bacterial growth under AgNPs was stopped, but it continued slowly. It appears that with the continuation of AgNPs treatment, the bacteria became resistant to the concentration used and was able to grow in the presence of AgNPs. The pattern of growth under the treatment of ultra-high diluted compounds showed a new structure. When a bacterium faced a different and toxic environment, it initially

changes its metabolism and produces a variety of oxidizing compounds, including aldehydes, ketones and alcohols. Tables 1 and 2 show, untreated bacteria volatiles are mainly hydrocarbons (>50%). From mentioned tables, it can be found that the AgNPs treatment resulted in the production of more than 80% of aldehydes, alcohols and ketones. It can hypothesize that the simple metabolite pathway transform into complex resistance metabolic pathway for exposure to acidic environments and survival at oxidative stress. Following Arg-n. treatment, the amount of hydrocarbons has decreased significantly and the amount of alcohol and terpenes have increased. The production of these two compounds shows that the metabolic pathways change into very complex and inefficient state, so that the bacteria cannot use extracellular monosaccharides and micronutrient. In such a situation, the bacteria have lost the ability to survive, grow and reproduce [13, 16, 23]. In a nut shell, the use of ultra-high diluted compounds initially increased growth and then decreased it. Also, in the continuous usage of these materials, no more bacterial growth was observed.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

Not applicable.

## FUNDING

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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