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Synergistic Effect of Silver and Zno Nanoparticles Green Synthesized by Vitis Vinifera Stem Extract with Ampicillin against Some Pathogenic Microbes

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Abstract

The increasing of multiple drug resistance pathogens necessitated extensive research for new safe and effective therapeutic agents. Vitis vinifera stem (VVS) hydroalcoholic extract beside its biosynthesized metallic nanoparticles silver (VVSAgNPs) and zinc oxide (VVSZnONPs) were tested against pathogenic microbes namely; B. subtilis ATCC6633, S. aureus ATCC29213, E. coli ATCC25922, S. typhimurium ATCC14028 and the fungus C. albicans ATCC1023. HPLC/MS/MS analysis of VVS extract led to identification of 31 compounds. The major compounds were malic, quinic and vanillic acids, catechin gallate and reservatrol and its dimer viniferin beside 11 fatty acid and terpene. The VVSAgNPs and VVSZnONPs were characterized using UV, FT-IR, TEM and zeta potential measurements. All the tested samples gave antimicrobial activity against all tested pathogens. VVSAgNPs were the most effective either alone or as synergists with the antibiotic ampicillin and giving the highest growth inhibition with the antibiotic at 1xMIC and 3xMIC. Also, the reduction of microbial population was more than 3 log for B. subtilis, E. coli and 2.3 log for C. albicans at 4xMIC using VVAgNPs. Thus, VVS hydroalcoholic can be used in food industry and pharmaceutical industry as antimicrobial agent while silver nanoparticles could be used as a promising disinfectant with long stability on surface materials that could be used in the hospitals as a disinfectant spray for preventing emergence of microbial resistance.

Keywords: Vitis viniferastem; silver nanoparticles; zinc oxide nanoparticles; pathogenic microbes; synergism

1. Introduction

Due to the antibiotic resistance which became a major problem over the world, thus it must be found an alternative safe and effective candidate to solve that problem. The natural extracts of V. vinifera parts contain several phenolic compounds which possess antimicrobial activity [1] and the production of different nanoparticles from the stem of this plant could present some great applications in drug delivery and medicine fields [2]. Silver nanoparticles were found to have good antimicrobial effect against many pathogens such as Staphylococcus aureus, Klebsiella pneumonia and Micrococcus luteus[3]. Furthermore, silver and zinc oxide nanoparticles have good advantage over the common antibiotics that is their mode of action is dependent on multiple processes in the cell so the probability of resistance emergence is lower than antibiotics [4, 5]. The mode of action include the interaction of these nanoparticles with the microbial cell wall or DNA in addition to production of reactive oxygen [6] and release of metal ion or interference with microbial replication [5].

Furthermore to enhance the effect of the common antibiotics; a synergistic interaction between them and plant extracts or their derived nanoparticles can be applied to overcome the microbial resistance and reduce the antibiotic dose consequently reduce its side effect [7]. This study aimed to evaluate the

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antimicrobial potential of Vitis vinifera stem extract and its ZnO and silver nanoparticles.

Experimental

Collection, identification and extraction of Vitis vinifera stem:

Vitis vinifera stem were collected from a private farm, Ashmoun, Menoufia, Egypt. The plant specimens were identified by Dr. Mohamed Elgebali, Consultant of botany, Orman garden, Giza, Egypt. Powdered material of VVS (0.5 Kg) were extracted by 75% (3 Ethanol/1 water) to yield 42 g of hydroalcoholic extract.

Profiling of secondary metabolites of Vitis vinifera stem using LC-MS/MS.

In this study, UPLC (the Acquity system, Waters, Milford, USA) hyphenated to Q-Exactive hybrid MS/MS quadrupole - Orbitrap mass spectrometer (Thermo, Bremen, Germany) was used. Chromatographic separation for this system was performed using deionized water acidified with 0.1% formic acid (solvent A) and acetonitrile (solvent B) with the BEH shield C18 column (150×2.1 mm, 1.7 µm). The mobile phase flow of 0.4 mL/min was adjusted to the following gradient: (0-14 min) from 5% B to 50% B, (14-20 min) to 98% B and kept in these conditions for 5 min. then system returned to the starting conditions and was re-equilibrated for 3 min. Q-Exactive MS was operated upon following conditions: the HESI ion source voltage (-3kV or 3kV). The sheath gas (N2) flow 48 L/min, auxiliary gas flow 13 L/min, ion source capillary temperature 250 °C, auxiliary gas heater temperature 380 °C. The CID MS/MS experiments were performed using collision energy of 15 eV [8]. Formulated mass spectral fragmentations of 33 phenolics, terpenes and other metabolites ion mobility tandem mass spectrometry (MSMS) were performed to provide a comprehensive fragmentation pattern with retention time and MSMS information. The four fractions, imported from raw MS data to export a common output 'Analysis Base File' (abf) format were packaged in MS-DIAL 4.60 (http://prime.psc.riken.jp/) [9]. This provided an enhanced standardized untargeted lipidomics and metabolomics by using (MSP) format libraries to filter noisy spectra via a classical spectral similarity calculation. The selected potential metabolites were searched and recognized by comparing their fragmentation patterns and retention indices (RI) with those available in Fiehn, MassBank, ReSpect, LipidBlast and RIKEN databasesand others[10, 11].

Synthesis of sliver nanoparticles (AgNPs)

10 ml of AgNO3 (1 mM) solution was reduced using hyroalcoholic extract of V. vinifera stem (300 µL) at room temperature. The mixture was shaken and allowed to stand at room temperature. The obtained colloidal solution was centrifuged at 4,000 rpm for 20 min followed by re-dispersion of the obtained nanoparticles in the deionized water. This process was repeated twice to isolate the pure AgNPs and to eradicate the unbounded plant extract residue [12].

Synthesis of zinc oxide nanoparticles (ZnONPs):

Synthesis of zinc oxide nanoparticles was undergo, 1.5 g of the hyroalcoholic extract of V. vinifera stem was mixed with 7.5 gm of zinc acetate (dissolved in one liter of distilled water) then heated at 80 °C for 20 min. Few drops of ammonia solution were added to the reaction mixture up to white colored precipitate was formed. The reaction mixture was left for 30 min for complete reduction to zinc oxide nanoparticles. Then the precipitate was centrifuged at 4000 rpm and washed two times by distilled water followed by ethanol to get off white powder by freeze drying [13]

Characterization of metal nanoparticles:

UV-Visible spectral analysis

Synthesis of silver and zinc oxide nanoparticles was monitored using a UV spectrophotometer model AGILENT Technology, Cary 100 UV-Vis spectrophotometer. The UV-Vis spectra were recorded between 250-500 nm.

FT-IR analysis:

FTIR 6100 spectrometer (Jasco, Japan) was used for determination of the functional groups of the synthesized nanometals and the VVS extract in the range of 4000–400 cm^{-1}.

Transmission electron microscopy (TEM)

The morphology and size of the reductive silver and zinc oxide nanoparticles was investigated by TEM (JEOL-JEM-1011, Japan). The samples were prepared by placing drops of the nanoparticles suspension on carbon coated copper grid, followed by allowing the solvent to evaporate slowly before recording the TEM image [14].

Zeta Potential Analysis:

Dynamic light scattering instrument (PSS, Santa Barbara, CA, USA)

used to estimate the zeta potential at 632.8 nm line of a He Ne laser as the incident light with an angle of 90◦ and the zeta potential with an external angle of 18.9◦ at room temperature [14].

Evaluation of antimicrobial potential and kinetics

1. Tested microorganisms

The antimicrobial potential of the hydroalcoholic extract, VVSAgNPs and VVSZnONPs were tested against three groups of microbes included gram positive bacteria such as Bacillus subtilis ATCC6633, Staphylococcus aureus ATCC29213, gram negative bacteria such as Escherichia coli ATCC25922, Salmonella typhimuriumATCC14028 and the fungus Candida albicans ATCC10231. All strains werekindly provided from Chemistry of Natural and Microbial Products, National Research Center. The strains were grown on nutrient agar and sabouraud dextrose agar slants for bacteria and fungi, respectively then kept at 4°C.

Inoculum preparation

The inoculum of the tested microbes was obtained from overnight cultures grown on the previously mentioned media at 37°C for 24h. After that, to reach the final concentration of approximately 106 CFU/mL, a loopful of the cultures was transferred into sterile saline solution then the turbidity was measured at 600 nm according to 0.5 McFarland scale tube.

Antimicrobial activity

Our tested samples (hydroalcoholic extract, VVSAgNPs and VVSZnONPs) were evaluated for their antimicrobial activities against the above mentioned microbes by agar well diffusion method [15]. Media were prepared and sterilized then cooled and poured in 9 cm Petri dishes containing 100 µl of inocula that prepared for each organism. After solidification of the media, wells of 10 mm were made and loaded with 100 µl of the different extracts (12.5 mg/ml). The plates were incubated at 37°C for 24 h then; the antimicrobial activities were determined by measuring the inhibition zone diameter (mm) against the standard antibiotics (ciprofloxacin and ketoconazole as antibacterial and antifungal, respectively) in a concentration of 0.5 mg/ml. Negative controls were prepared using DMSO applied for diluting the samples.

Minimal inhibitory concentration (MIC)

The MICs were detected using serial broth macrodilution method[16]. In this test, 100 µl of the inocula obtained from the above step were added to one ml of the culture media containing different concentrations of the tested samples which firstly dispersed in DMSO to reach 105 CFU µg/mL. The concentrations ranged from 5 to 1000 µg/ml. Triplicate tests were performed and the average was taken as the final reading comparing to negative and positive controls.

Evaluation of synergistic effect

The synergistic effects between the antibiotic Ampicillin and hydroalcoholic extract, VVSAgNPs and VVSZnONPs were evaluated by agar well diffusion method [15]. Different combinations with different concentrations were made between the antibiotic, hydroalcoholic extract, VVSAgNPs and VVSZnONPs. The dilutions of the antibiotic to the tested samples were 100 : zero, 75 : 25, 50 : 50, 25 : 75 and zero : 100 compared to 75%, 50% and 25% of the antibiotic alone as a positive control or plant alone as negative controls. The antibiotic stock concentration started with 20 µg/mL and they started concentrations of the three plant extract samples were 1xMIC and 3xMIC. After loading the wells with the previous dilutions; the plates were incubated at 37°C for 24 h then the inhibition zone diameter was measured (mm).

Kill time analysis using different concentrations of prepared nanoparticles.

To study the kill time of the three tested microbes; different concentrations of zinc and silver nanoparticles of our extract were applied. The freshly prepared inocula (105cfu/ml) were inoculated in tubes containing different concentrations of samples (1×MIC, 2×MIC, and 4×MIC) diluted in 5 ml phosphate buffer saline and incubated at 37°C. Samples were taken at 0, 30, 60 and 120 min then serially diluted and plated in the respected agar plates to detect the viable cell counts after 24h at the same temperature. Both of the positive and negative control was tested at the same conditions [17].

Results and discussion

UV Spectroscopy:

Synthesis of the V. vinifera stem silver and zinc oxide nanoparticles was monitored by Ultraviolet spectroscopy (UV) at a wavelength range of 200 to 500 nm. The changing of silver nitrate color after addition the extract from clear solution to dark brown was observed. The dark solution of VVSAg mediated

nanoparticles was showed characteristic UV absorption peak at 419 nm due surface plasmon resonance phenomena (SPR) of the electrons [18] while Zinc oxide nanoparticles prepared using V. vinifera stem hydroalcoholic extract showed UV absorption peak λmax at 330 nm which is characteristic for zinc oxide nanoparticle which showed shorter wavelengths absorptions [19]. **(Figure 1)**

Infrared spectroscopy:

FT-IR spectrum of the V. Vinifera stem hydroalcoholic extract powder, Zinc oxide and silver nanoparticles in the spectral width range from 400 to 4000 cm^{-1} (Figure 2). It showed peaks at 3421, cm^{-1} attributed to the O-H stretching mode of the total extract with high intensity. It was observed the decrease of the peak intensity in case of ZnONPs and AgNPs which interprets the role of phenolic hydroxyl moieties in the reduction of both silver nitrate and zinc acetate to their metallic nanoparticles. The characteristic bands corresponding to the ZnO nanoparticles stretching mode are observed at 445.5 and 878.4cm^{-1}[20].

Transmission Electron Microscopy:

Transmission Electron Microscopy investigation of VVZnONPs [Figure 3 (A)] showed hexagonal nanoscaled particles of about 100 nm and consists of small nanoparticles aggregates with dimensions ranging from 11 to 28 nm diameter. On the other hand; TEM of VVAgNPs (showed spherical shaped nanoparticles with dimensions ranging from 13 to 18 nm diameter. Also, the effect of hydroalcholic extract in capping and stabilizing the prepared silver nanoparticles is appear clearly in Figure 3 (B).

Zeta potential analysis:

Zeta potential analysis was measured for VVSZnONPs and VVSAgNPs for examination of its stability during dispersion in the aqueous suspensions. The VVsZnONPs showed zeta potential at – 29.49 mV while VVSAgNPs were measured at -95.74 mV, which in turn reflects the very high stability of silver nanoparticles suspensions (VVSAgNPs) compared to ZnO nanoparticles (VVZnONPs).These results clarified that VVSAgNPs can maintains their suspensions over long-term and clearly indicate the successful formation of would be useful for biological and industrial aspects [21]. **(Figure 4)**

Figure 5: LC-MS/MS chromatogram of Vitis vinifera stem.

The metabolome of hydro-alcoholic extract of Vitis vinifera stem (VVS) was analyzed and compared with previously reported data led to identification of 31 compounds. A combination of both ionization modes (positive and negative) in MS full scan mode was applied for the molecular mass determination of the compounds in hydro-alcoholic VVS. Compounds identification was performed by comparing the observed m/z values and the fragmentation patterns with the literature. The list of compounds identified in the hydro-alcoholic extract of VVS is represented in Table 1. The 31 compounds shown in figure 5 belong to different natural products families including organic acids such as quinic acid and malic acid. These two compounds are major in the plant extract. In addition to, phenolic acids [22] such as gallic acid, protocatechuic acid, resorcylic acid, gentistic acid, p-hydroxybenzoic acid, calicylic acid, p-coumaric acid, dihydroxyphenyl acetic acid, ferulic acid and sinapic acid among them vanillic acid was major in the VVS plant extract. Moreover, condensed tannins such as catechin gallate was major. The most predominant compounds in the extract was the stilbene metabolite resveratrol and its dimer viniferin. In addition, 11 Fatty acid and triterpenes compounds were also identified in analyzed VVS samples [23].

Antimicrobial activity

The antimicrobial activity of the tested samples was detected against some common pathogens such as B. subtilis ATCC6633, S. aureus ATCC29213, E. coli ATCC25922, S. typhimurium ATCC14028 and the yeast C. albicans ATCC10231 (Table 2). All samples inhibited the growth of the tested microbes with different degrees [24] where. S. aureus was the most sensitive ones and C. albicans as well asS. typhimurium were more resistant and possessed the lower inhibition zone [25]. This may be due to that Vitis vinifera stem extract is mainly consists of phenolic compounds such as reservatrol and its dimer vinferin beside some acids such as quinic, malic and vanillic acids which have antimicrobial activity (Adamczak et al. 2020) by affecting the cell membrane permeability causing cell constituents

leakage [26]or enhance the cleavage of essential enzymes in the cell consequently cell death such as rutin (Bernard et al., 1997) and can inhibit DNA gyrase enzyme by the presence of quercetin [27]. These phenolic compounds have an advantage over the synthetic chemical medicines as no hemolytic activity against human blood cells [28] which make them suitable for medical applications [29].

Furthermore, our results indicated that silver nanoparticles were the most effective ones compared to other samples or reference antibiotic. The mode of action of VVSAgNPs may rely on the formation of pores in cell wall depending on the particle size, where the small NPs can bind with the DNA or protein of the cell causing death [30] or may affect the cell permeability [31]. Finally, the effect of different plant forms was acting in a dose dependent manner. The presence of resveratrol and viniferin gave high activity against Candida albicans.

Minimum inhibitory concentration

Our results presented in Table 3 revealed that there was different sensitivity of tested organisms towards the plant extract and its NPs with different MIC values (8-600 µg/ml). In this context, [32] obtained similar results usingEscherichi coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumoniae and L. monocytogenes with MIC ranged from 66 to 134 mg/ml. While, [33] found different MICs this may be due to using of different extraction method which affect the phenolic profile of the plant [34].

Also, it was noticed that gram positive bacteria and fungi were more susceptible towards vitis extract and its NPs than gram negative which has higher MIC concentration [35]. This may be attributed to that gram negative bacteria possess a strong hydrophillic cell membrane consists of two layers, while there was a simple membrane in gram positive bacteria which allows the passage of lipophilic compounds [36]. Also, the cell walls of gram negative bacteria have a lipo-polysacharide layer not found in gram positive ones [37].

Silver NPs were found to have the strongest effect against the tested microbes indicated by the smallest MIC (8 µg/ml for B. subtilis, C. albicans and 25 µg/ml for E. coli). This may be due to that their mode of action depended on their large surface area that ease the interaction between metal nanoparticles and microbial cell membrane [38].

Synergistic effect between plant extracts and antibiotics

The plant extract or its NPs may act as antimicrobial agent alone or may act as antibiotic resistance modifying agent by combination with antibiotic and increase its effectiveness against the pathogenic microbes, also can minimize its MIC which reduces its side effects [39]. Table 4 shows the synergistic effect between the antibiotic Ampicillin and VVS in addition to its metallic NPs against the three selected microbes. Our results revealed that all plant forms at the applied concentrations improved the efficacy of Ampicillin against the tested pathogens compared to each of them alone [40]. The highest synergism was found under the effect of silver NPs. The ability of plant extract to enhance antibiotic activity may be attributed to increasing permeability, drug efflux inhibition and inhibition of β-lactamase [41].

Kill-time analysis

To detect the kill time analysis of vitis NPs in relation to cell viability of the three tested microbes; different concentrations of silver and zinc oxide NPs (1xMIC, 2xMIC and 4xMIC) were applied (Figure 6). Data showed that the microbial population reduced by increasing both of concentration and incubation time indicating that the bactericidal or bacteriostatic effect acting in a dose dependent manner [1]. VVSAgNPs showed bactericidal effect with the tested bacteriawhere the reduction rate was more than 3 log CFU/ml, in contrast to C. albicans which had lower reduction value (2.3 log CFU/ml). On the other hand, VVSZnONPs had lower viable cell count (0.8, 1.8, 1.7 log CFU/ml for B. subtilis, E. coli and C. albicans, respectively) indicating its bacteriostatic and fungi-static effect after 2 hours of incubation [42]. Suggesting that VVSAgNps mode of actions depended on From the previous results however, E. coli had the highest MIC but its population decreased and reached the kill point after 2 hours, this suggests that the time needed to kill can differ from organism to another (Xia et al., 2010) or the different mode of actions of the applied material is dependent on the organism type [43].

Conclusions

The Vitis vinifera stem (grape stem) is an agro waste from grape industry. The hydroalcoholic

extract of grape stem are rich in phenolic constituents, fatty acids and organic acids. It exhibited potent antimicrobial against different pathogenic microbes. Both grape stem extract, and its synthesized metallic nanoparticles (Ag and ZnO) are potent antimicrobial agents, while, The VVSAgNPs was more potent than VVSZnONPs, extract and the standard drugs. The potency of VVSAgNPs is might be due to the unusual high stability of its silver nanoparticles as suspension in the liquid (it is estimated by Zeta Potential analysis at -95mV). The VVSAgNPs seemed to be good candidate to be a potent disinfectant spray possesses ecofriendly, environment friendly and safety for application in medical industry and hospitals medical care rooms.

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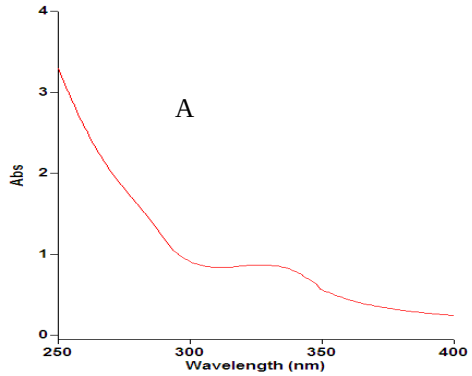

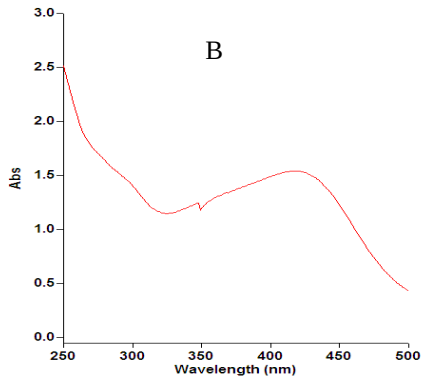

Figure 1: UV spectrum of VVSZnO (A) and VVSAg (B) nanoparticles

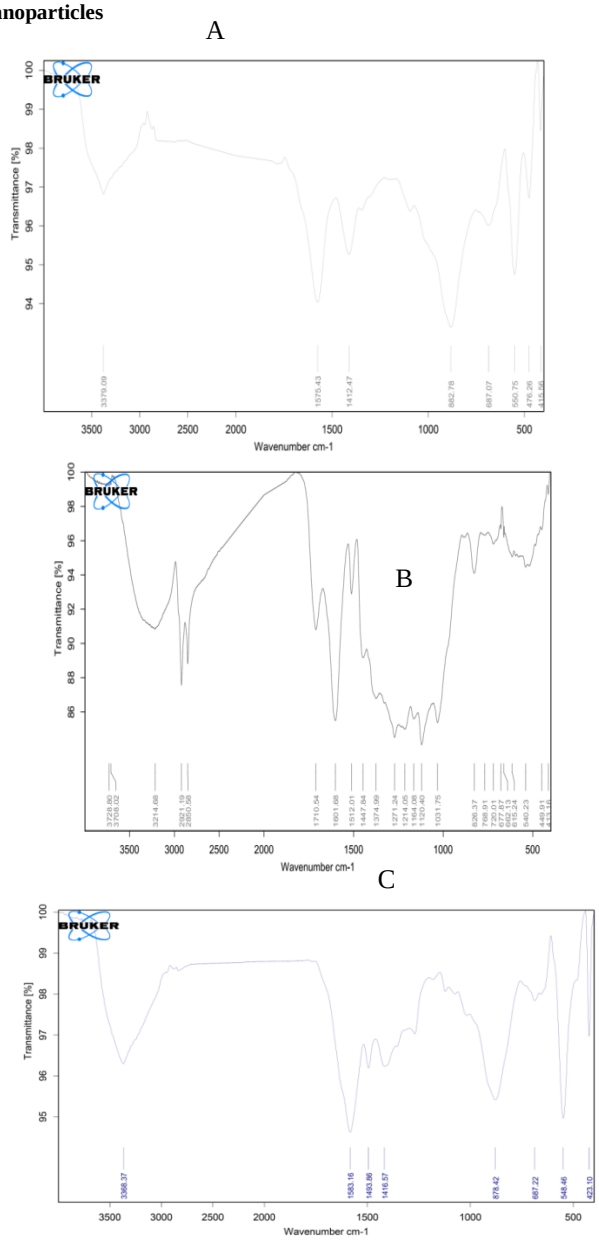

Figure 2: FT-IR of VVS extract (A), VVSAg (B), VVSZnO (C)

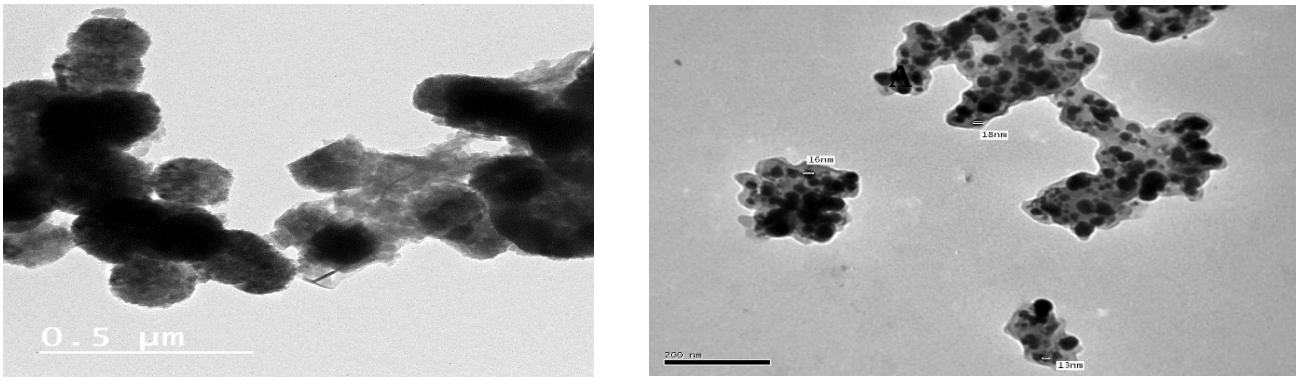

Figure 3: TEM image of VVZnONPS (A) and TEM Image of VVAgNP (B)

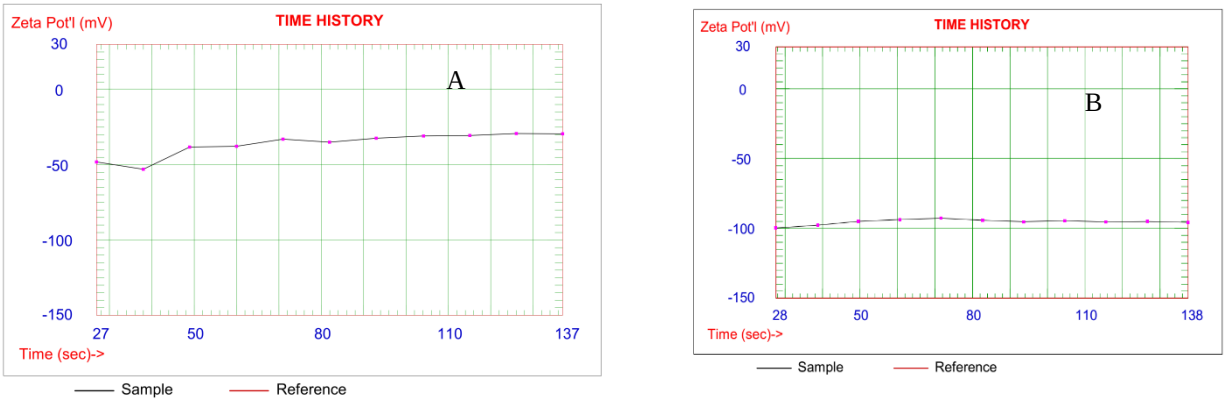

Figure 4: Zeta potential of VVSZnO NPs (A) and VVSAgNPs (B)

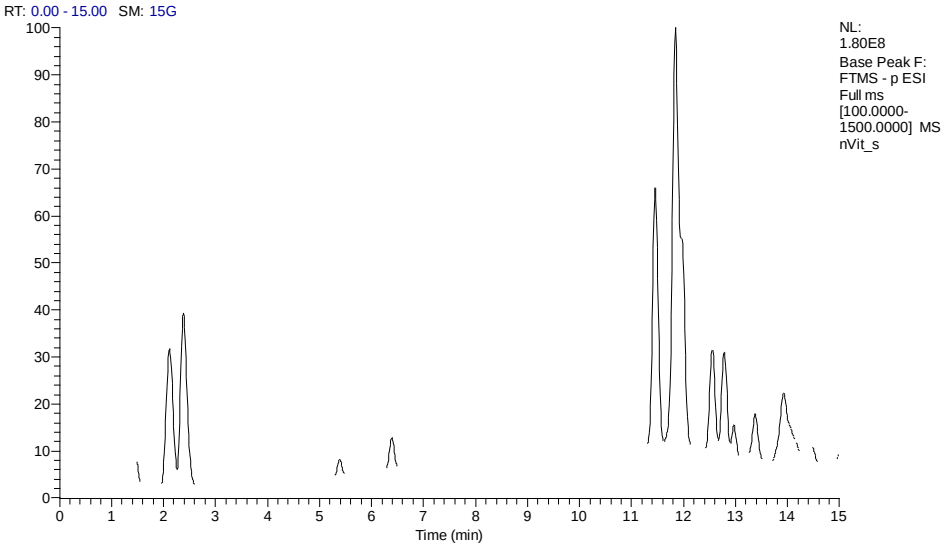

Figure 5: LC-MS/MS chromatogram of *Vitis vinifera* stem.

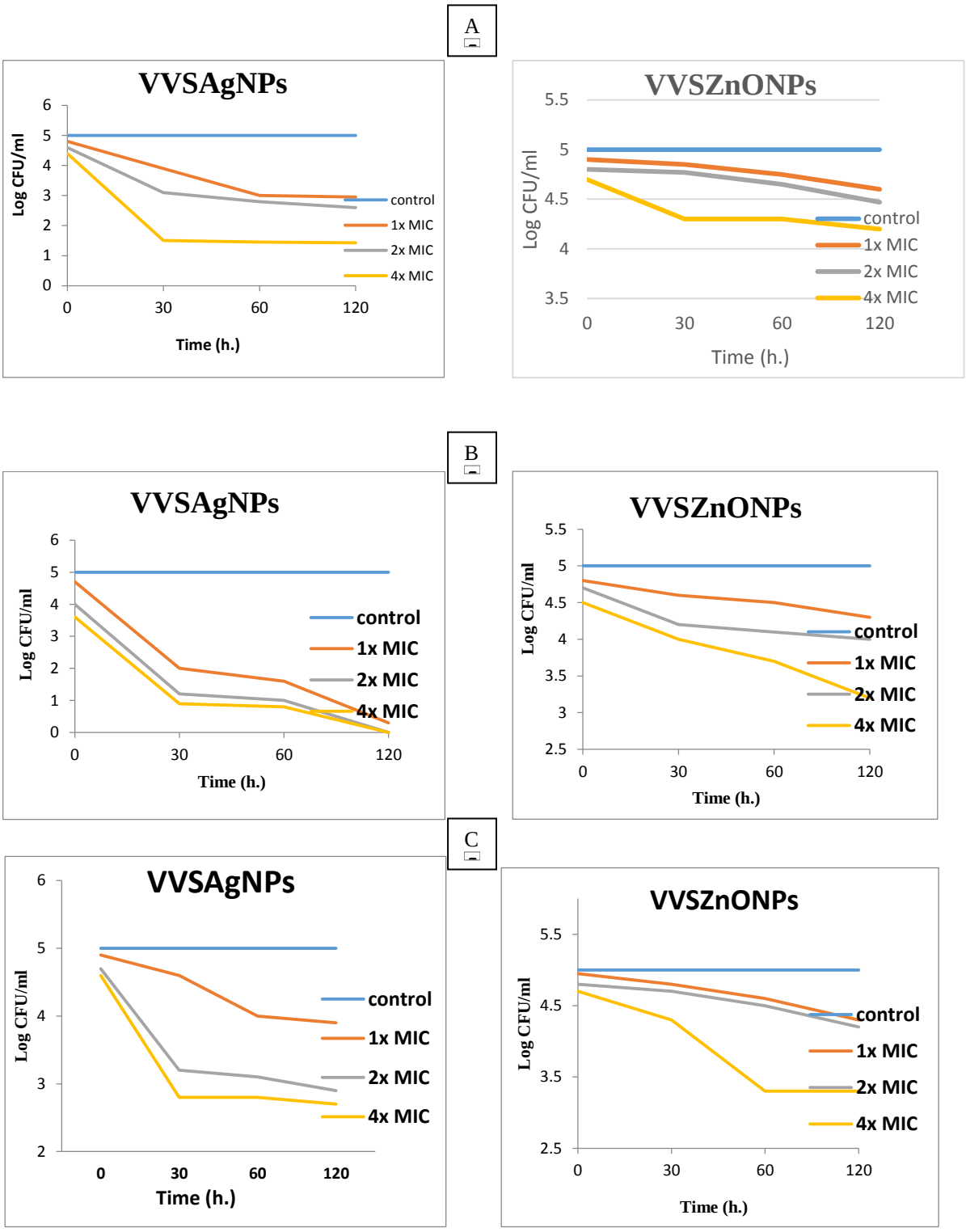

Figure 6: Time-kill effect of *Vitis vinifera* stems' Ag and ZnO nanoparticles applying different concentrations of MICs on A) *B. subtilis* ATCC6633, B) *E. coli* ATCC25922, and C) *C. albicans* ATCC10231

Table 1: Phenolic constituents and other compounds from *Vitis vinifera* stem

No	RT [Min]	metabolite identification	Chemical formula	[M-H]⁻ Measured and calculated	[M-H]⁻ Fragmentation	[M-H]⁺ measured	[M-H]⁺ Fragmentation	Δ ppm
Phenolic constituents								
1	2.15	Quinic acid[a]	$C_7H_{12}O_6$	191.0552, 191.0550	111.0072	193.0495, 193.0495	133.0286	1.1636
2	2.40	Malic acid[a]	$C_4H_6O_5$	133.0128, 133.0131	115.0021, 89.0229, 71.0122	135.0443, 135.0441	107.0860	-2.7282
3	2.80	Gallic acid[a]	$C_7H_6O_5$	169.0129, 169.0131	125.0229			1.2443
4	3.59	Protocatechuic acid[a]	$C_7H_6O_4$	153.0181, 153.0182	109.0280	155.0702, 155.0703	109.0655, 81.0707	0.9033
5	3.62	Resorcylic acid[a]	$C_7H_6O_4$	153.0179, 153.0182	109.0279, 136.2530, 78.9574			-1.9005
6	3.77	Gentistic acid[a]	$C_7H_6O_4$	153.0181, 153.0182	109.0279, 81.0327	155.0343, 155.0339	137.0231, 111.0445, 81.0705	-1.2025
7	3.92	p-Hydroxybenzoic acid[a]	$C_7H_6O_3$	137.0230, 137.0233	93.0330	139.0393, 139.0390	111.0445, 93.0340	-2.1532
8	4.48	Salicylic acid[c]	$C_7H_6O_3$	137.0232, 137.0233	93.0330	139.0390, 139.0390	93.0704	-0.5942
9	5.28	Vanillic acid[a]	$C_8H_8O_4$	167.0338, 167.0339	152.0103, 124.0148, 111.0068	169.0497, 169.0495	151.0391, 125.0599, 111.0445	-0.7035
10	5.40	*p*-coumaric acid[ac]	$C_9H_8O_3$	163.0390, 163.0391	119.0487	165.0907, 165.0910	119.0494	-1.5880
11	5.46	Dihydroxyphenyl acetic acid[b]	$C_8H_8O_4$	167.0339, 167.0339	152.0102, 139.0023, 124.0150, 95.0123	169.0497, 169.0495	155.0340, 137.0235, 125.0599	-0.4295
12	6.06	Ferulic acid[ab]	$C_{10}H_{10}O_4$	193.0498, 193.0495	178.0261, 149.0597, 139.0386, 134.0359	195.1378, 195.1380	177.1273, 149.1329, 135.1168	1.4746
13	6.07	Sinapic acid[bc]	$C_{11}H_{12}O_5$	223.0622, 223.0601	208.0370, 193.0126, 164.0467, 149.0230	225.1296, 225.1274	207.0650, 192.0417, 147.0440	9.2547
14	6.39	Catechin gallate[b]	$C_{22}H_{18}O_{10}$	441.0830	289.0718, 245.817, 203.0688			3.122
15	6.47	Quercetin-3-O-rutinoside[b]	$C_{27}H_{30}O_{16}$	609.1943	301.0716	611.1981, 611.1982	303.0862	
16	11.45	Resveratrol[c]	$C_{14}H_{12}O_3$	227.2010, 227.2006	183.1379, 165.1276	229.1434, 229.1434	183.1168, 109.1017	1.9012
17	11.74	Viniferin[c]	$C_{28}H_{22}O_6$	-	453 [m+HCOOH]=499, [2m+1]= 907	455.1164, 454.1184	427.1112, 371.8875, 242.0306	-4.4130
18	12.49	Ursolic acid[a]	$C_{30}H_{48}O_3$	455.3367, 455.3375	393.3383, 261.0401, 211.4946			1.7763
19	12.34	16-Hydroxyhexadecanoic acid[c]	$C_{16}H_{32}O_3$	271.2278, 271.2268	253.2175, 225.2219			3.9557
Fatty acid and triterpenes class								
20	3.05	rac-Glycerol 3-phosphoate[d]	$C_3H_9O_6P$	171.0052, 171.0053	96.9682 [H2O4P], 78.9574[O3P]	173.0813, 173.0808	98.9847[H4O4P], 90.9482	-0.8390
21	4.27	Pimelic acid[d]	$C_7H_{12}O_4$	159.0649, 159.0652	141.0543, 130.9817, 115.0750, 97.0643	161.0820, 161.0808	145.1337, 133.0651	-1.5335
23	6.28	Azelaic acid[d]	$C_9H_{16}O_4$	187.0967, 187.0965	169.0857, 125.0957, 97.0640	189.1121, 189.1121	125.0965, 97.1017	1.2013
24	7.39	Sebacic acid[d]	$C_{10}H_{18}O_4$	201.123, 201.1121	183.1015, 139.1114	-	-	0.6895
25	11.42	FA 18:4+1O[d]	$C_{18}H_{28}O_3$	291.1961, 291.1955	247.2065, 185.1170, 125.0956	293.2109, 293.2111	275.1995, 247.2067, 165.1274	2.1798
26	11.84	Linoleic acid[d]	$C_{18}H_{32}O_2$	279.2325, 279.2319	96.9585	-	-	2.3518
27	1 1.90	beta-Hydroxymyristic acid[d]	$C_{14}H_{28}O_3$	243.1962, 243.1955	197.1903, 174.9550, 59.0122	245.2264, 245.2264	175.1479	3.1747
28	12.25	Oleic acid[d]	$C_{18}H_{34}O_2$	281.2486, 281.2475		283.2628. 283.2632	-	2.7649

29	12.79	9-Trans-Palmitelaidic acid[d]	$C_{16}H_{30}O_2$	253.2169, 253.2162	-	-	-	2.8129
30	12.88	Stearic acid[d]	$C_{18}H_{36}O_2$	283.2643, 283.2632	-	285.2896, 285.2788	-	3.9729
31	13.41	Palmitic Acid[d]	$C_{16}H_{32}O_2$	255.2327, 255.2319	237.1713	-	-	3.3501

- Chemical formulas were calculated on the basis of accurate masses measured in HESI-MS/MS, and fragmentation pathways were described on the basis of ESI-$MS^n$with those of the standard compounds, data available in PubChem, MS-Dial, Metlin, KNApSAck databases, results of own previous analysis and literature data.

- Superscript Letter a,b) represent tentative identification means compared by this literature (Püssa, Floren et al. 2006, Jaitz, Siegl et al. 2010, Razgonova, Zakharenko et al. 2021) then letter c) was compared by (Barros, Gironés-Vilaplana et al. 2014) and letter d) for fatty acids (Pérez-Navarro, Da Ros et al. 2019).

Table 2: Antimicrobial activity of plant extract and nanoparticles (12.5 mg/ml) expressed as inhibition diameter zones in millimeters (mm)

Treatment type	Inhibition zone diameter (mm)				
	Gram +ve		Gram –ve		Fungi
	B. subtilis	*S. aureus*	*S. typhimurium*	*E. coli*	*C. albicans*
Vitis vinifera stem (VVS) extract	19	20	11	18	15
VVSZnONPs	17	20	14	16	15
VVSAgNPs	22	25	19	18	19
CFL	16±2.0	15±0.5	19±2.0	18±1.5	-
KET	-	-	-	-	16±0.5

CFL = Ciprofloxacin KET = Ketoconazole

Table 3: Minimum inhibitory concentration (µg/mL) of extracts against the pathological organisms

Treatment type	Concentration (µg/ml)		
	Gram +ve	Gram –ve	Fungi
	B. subtilis	*E. coli*	*C. albicans*
VVS extract	250	600	25
VVSZnONPs	125	250	125
VVSAgNPs	8	25	8
CFL	12	13	-
KET	-	-	12

CFL = Ciprofloxacin KET = Ketoconazole

Table 4: Synergistic effect of antibiotic/Vitis vinifera stem extract, VVZnO and VVAg nanoparticles

Treatment type at MIC concentration (µL/ml.) against Ampicillin (Amp) at 20 µg/ml	Inhibition zone diameter (mm) Gram +ve	Gram –ve	Fungi
	B. subtilis	*E. coli*	*C. albicans*
Ampicillin 100%	33.0	24.0	33.0
75%	30.0	20.0	30.0
50%	28.5	17.0	29.5
25%	26.5	15.0	27.5
VVS extract at 1xMIC (25-100%)	16	15	17
at 3xMIC (25-100%)	16	15	18
VVSZnONPs at 1xMIC (25-100%)	16	15	17
at 3xMIC (25-100%)	16	15	18
VVSAgNPs at1xMIC (25-100%)	16	15	17
at 3xMIC (25-100%)	18	15	18
Amp + 1xMIC V. extract			
3:1	34	21	32
1:1	33	21	30
1:3	27	17	28
Amp + 3xMIC V. extract			
3:1	35	26	33
1:1	35	21	30
1:3	29	17	28
Amp + 1xMIC Zn. Nanoparticle			
3:1	31	21	32
1:1	30	21	30
1:3	28	20	28
Amp + 3xMIC Zn. Nanoparticle			
3:1	34	21	34
1:1	35	21	32
1:3	29	20	28
Amp + 1xMIC Ag. Nanoparticle			
3:1	34	26	34
1:1	35	22	32
1:3	29	18	29
Amp + 3xMIC Ag. Nanoparticle			
3:1	34	26	34
1:1	35	23	32
1:3	30	20	31