Original Research Article

Antibacterial effect of biosynthesized zinc oxide nanoparticles against *Erwinia amylovora* and induction of crop growth.

Abstract:

Objectives: Zinc oxide nanoparticles (ZnO-NPs) are widely recognised as one of the most promising types of materials in a wide range of applications, including agriculture. Modern systemic efforts have identified several therapeutically active microalgae derived compounds, including phenols, flavonoids and others. The antibacterial properties of the phenolic substances were demonstrated. Hence, the present study aims to exhibit the antibacterial activity of the bioactive compound capped silver nanoparticles in *in vitro* condition.

Methods: Bio active compound separated by Solid phase Extraction method. Dispersible Zinc oxide nanoparticles synthesized using bioactive compound as the major capping agent. Zinc nitrate was used as starting material and its reduction was carried by phenolic components of *Spirulina platensis* aqueous extract from $Zn2+$ to ZnO. The synthesized Zinc oxide nanoparticles characterized by H^1 NMR spectroscopy. Conjugated nanoparticles characterized physically by SEM analysis. Scanning Electron Microscopy (SEM) demonstrated particle sizes in the range 10–15 nm. ZnO nanoparticles demonstrated antibacterial activity against an isolated plant pathogen *Erwinia amylovora*. Time kill determination assay were done.

Findings: Phenols obtained after Solid Phase Extraction. Hence, this was regarded as the maximum quantified bioactive compound of *Spirulina platensis*. H_1 NMR spectroscopy analyses showed the presence of phenolic compounds and alcohols groups of long chain were also detected. In SEM analysis, the mean diameter of spherical Phenols-ZnONPs is less than 15 nm surrounded by the capping agent. In given time periods of 4, 8, 16, and 24 hour cells, concentrations of 1000µg/mL were 42 %, 33 %, 20 %, and 18 %. At 500 µg/mL of extract concentration, *Spirulina platensis* inhibited 50% bacterial proliferation (IC50) of *Erwinia amylovora*. A significant inhibitory effect ($p<0.0001$) was seen against the plant pathogenic strain.

Novelty: In addition to their antibacterial activities, biosynthesized ZnO-NPs are thought to show promise efficacy as growth accelerators. The most dangerous bacterial disease of pear and apple trees is fire blight, caused by *Erwinia amylovora*. Phenolic capped ZnO-NPs have been found to be efficient plant pathogen antagonists.

Keywords: Zinc oxide nanoparticle, *Spirulina platensis,* X ray diffraction, Scanning electron microscope, Phenols, *Erwinia amylovora* and Fourier transform infrared spectroscopy.

Introduction:

Metal oxide (MNPs) may be produced in a variety of ways (chemical, physical, and biosynthetic) and have a diverse set of characteristics and uses. Green synthesis covers the synthesis from algae, fungus, plants, bacteria, and other microorganisms. They enable the large-scale manufacturing of ZnO-NPs devoid of contaminants. Microalgae have been utilized to generate ZnO-NPs because of the specific biocompounds they produce. Natural extracts of Algal components offer a low-cost and environmentally beneficial alternative to using intermediary base groups. (1)

Secondary Algal compounds found in crude algal extracts function as both reducing agents and capping or stabilizing agents. Metal ions or metal oxides are reduced to zero valence metal NPs in bioreduction with the aid of Algal-secreted biocompounds such as polyphenolic and phenolic compounds, alkaloids, polysaccharides, amino acids, vitamins, and terpenoids. (2)

Algae also have the ability to grow without the help of any addition of outside chemicals or fertilizers. Microalgae grow extremely quickly and, on average, double their mass 10-fold faster than higher plants. It is known that various species of microalgae reduce metal ions. The results clearly showed that the size of produced NPs reduces as the content of an algal extract increases. All tests revealed NPs with spherical and hexagonal disc shapes, as verified by XRD and SEM analyses. Furthermore, these synthesized NPs were also proved efficient in various biomedical applications like antimicrobial, anti-cancer, anti-diabetic, and antioxidants. (3)

Recently, there has been a lot of interest in microalgae as a source of new, physiologically active compounds such phycobiline, phenols, terpenoids, steroids, and polysaccharide. However, the presence of phenolic chemicals in blue green algae is less well established than in higher plants. Algal phenolic chemicals have been identified as a possible contender for combating free radicals, which are detrimental to human bodies and plant systems. Manipulation of growing conditions for biomass production and productivity, on the other hand, is commonly utilised in the commercial manufacture of potentially valuable chemicals such as carotenoids and phenolics. (4)

Here, we disclose the bio-assisted synthesis of ZnO-NPs through an ecofriendly approach using aqueous extracts of *Spirulina platensis* as an efficient oxidizing/reducing and capping agent. The biosynthesis of ZnO-NPs has already been reported; however, their diverse biological properties including antibacterial activities in plants have been less exposed. The aim of the present study is therefore to investigate the biological effects of phenolic capped ZnO-NPs. H NMR spectroscopy and SEM were used to characterize the ZnO-NPs. The well-characterized ZnO-NPs were examined for their biological activities in plant pathogen. The *in vitro* antibacterial potential against *Erwinia sps* was examined for their possible application in the crop field.

Materials and Methods:

The experimental organism *S. platensis* was isolated from Kodai road, Dindugal, Tamilnadu (India) and cultivated in Zarrouk's medium (5) under 30±2°C temperature and illuminated with white fluorescent lamps at a light intensity of 2,000 lux (Sharma et al 2014).

Zinc nitrate and other reagent were purchased from Sisco Research laboratory, Chennai.

Preparation of microalgal Extract

In an Erlenmeyer flask, 5 g (dry weight) *S. platensis* biomass was suspended in 100 ml double distilled sterile water and heated for 15 minutes at 100°C. (5) The mixture was boiled, then cooled and centrifuged for 15 minutes at 10,000 rpm. The supernatant was collected and kept at 4 degrees Celsius for further analysis.

Solid Phase Extraction:

Solid phase extraction [phenols from *Spirulina platensis*] is carried out and the filtrates are collected in a petriplate each of 10 ml quantity and the solvent is evaporated at atmospheric pressure in water bath. After drying the residual weight is noted down and subtracted from the initial empty weight of the petriplate, and the amount of phenolic compounds being extracted is calculated. (6)

H^1 NMR spectroscopy

To analyze the BAC composition and purity within the limits of detection of the method.

Equipment:

^{1}H NMR spectrometer (e.g., Bruker-400 (400 MHz) with Avance II console and Top-spin processing software) (7)

Procedure:

The samples were dissolved through dimethyl sulfoxide-d6 (DMSO-d6) as a solvent. A volume of 20 μL of tetramethylsilane (TMS) was added as the internal reference. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) expressed in δ units, and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), or m (multiplet).

Synthesis of bio active compound capped silver nanoparticles:

BAC from *Spirulina platensis* was dissolved in 1 mL methanol, and 50 mL volume was made up with deionized water to make a stock solution of 5 mM. BAC – conjugated Zinc oxide nanoparticles were synthesized by the reduction of Zinc nitrate solution using sodium borohydride in the presence of phenols. One milliliter of 1 mM Zinc nitrate solution was magnetically stirred with 1 mL of 1 mM phenols before 5 μL of freshly prepared 5 mM sodium borohydride was added and stirring was continued for 2 h. (8)

Physical analysis - SEM analysis:

SEM experiments were performed to characterise the size and shape of bio-reduced Zinc oxide nanoparticles. Purified nanoparticles were sonicated for 15 min to make it uniform distribution and SEM measurements were performed at Maduari Kamraj university, Madurai. A samples was placed on the sample holder and was scanned at a magnification of 17000x, 28000x, 40000x, 50000x and 60000x. (9)

In vitro Antimicrobial Assay of ZnONPs Against Multidrug-Resistant Bacterial Strains :

The weakness of ZnONPs against multidrug-safe bacterial strains was resolved utilizing Kirby-Bauer's plate dissemination and agar well dispersion technique as per CLSI (Clinical Laboratory Standard Institute) Guidelines (2009). Bacterial strain *Erwinia sps* was utilized. About 50μL of the test (grouping of ZnONPs 2 mg/50 μL DMSO) was utilized against each strain cleaned on supplement agar plates followed by brooding at 37°C for 24 hrs. DMSO was utilized as negative control while 50μL watery concentrate of *S.platensis* was utilized as sure control. Antibacterial activity was assessed using a zone of inhibition (ZoI) measured after the incubation period against tested bacteria.

Time–kill determination

To investigate possible antibacterial activity and thus minimise potential toxicity and resistance problems, mixtures of nanoparticles were also tested. ZnO at sub-MBC concentrations were used in killing assays against *Erwinia sps.*

At time zero, ca. 5×107 CFU/mL of bacteria was added to the nanoparticle suspension at a dilution of 1 in 80. Incubation was then carried out in a shaking incubator (200rpm at 37 ◦C in air for up to 4 h) Inoculated nanoparticle-free suspensions in PBS were used as negative controls. Growth was assessed by plating serial dilutions of each nanoparticle/bacterial suspension at different time points onto tryptone soya agar plates. Plates were then incubated at 37 ◦C in air with CO2 for 24 h. [10]

Influence of biosynthesized ZnO-NPs on *Vicia faba* seed germination

The influence of biosynthesized ZnO-NP concentrations on the percentage of germination in the various concentrations over five days. 50 micro litre of *Erwinia sps* was inoculated in the soil.

Statistical Analysis

The examination was acted in sets of three, Mean ± SEM (S.D) was contrasted and the standard utilized and dissected measurably utilizing chart paid instat Dataset1, One way ANOVA. Estimations of $p < 0.05$ were considered statistically significant. IC10 and IC50 esteems in addition to their 95% certainty spans were determined utilizing the probit examination in SPSS variant 13.

RESULTS:

Phenolic extraction:

Solid phase extraction:

Phenolic chemicals are separated from the crude extract of *Spirulina platensis*. However, there are a variety of different chemicals, such as alkaloids produced in larger proportion. As a result, the crude extract was shaken with water. After solid phase extraction, alkaloids dissolved in water and phenolic rich fraction are obtained.

Isolated Bio Active Compound [BAC] where further confirmed by H^1 NMR spectroscopy

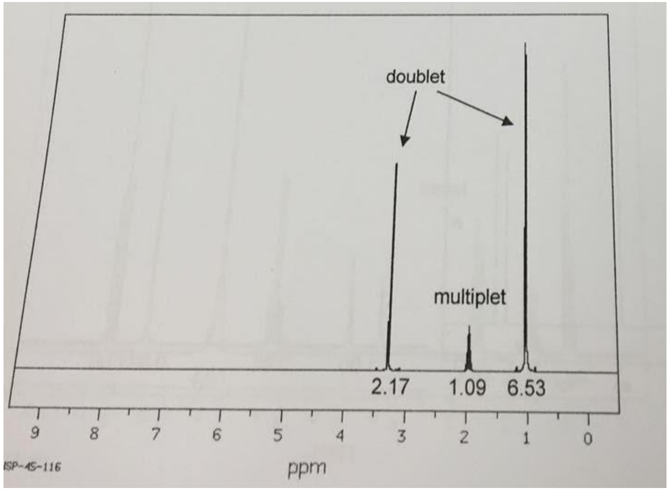
H^1 – NMR spectroscopy:

Reactions of bio active compounds were carried out in the presence of chloroform. All the reaction mixtures were studied by 1 H - Nuclear Magnetic Resonance Spectroscopy (1 H-NMR). Differences in the composition of the reaction mixture of the BAC by 1H-NMR analysis were found. During the solid phase extraction, phenols found as major products.

We came to know the highest peak [2977.7] was phenols. Hence solubilization was done with water because water is a poor solvent to dissolve alkaloids. Though other compounds were referred to simultaneously and show no specific pharmaceutical activities. Therefore, phenols was separated further and again checked for nmr reading. Comparing the 1H-NMR spectra of phenols before and after solubilization meaningful differences were found (Graph: 2). The phenolic spectrum only showed the corresponding signals of the protons with chemical shifts between δ 2.13-6.5 ppm and the corresponding signals of the rest of the protons, below δ 2.0 ppm showed other compounds. On the other hand, 1H-NMR spectra of soulbilized mixtures obtained under all the experimental conditions described above were identical (from a qualitative point of view).

In graph: 2, a representative spectrum of phenol is shown. Finally, we found that phenol is a bioactive compound and it is capable of interacting with the metal ions, by inter and intramolecular reactions.

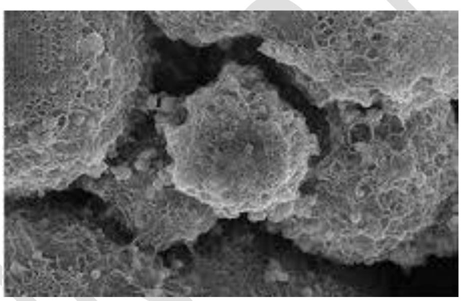
Graph: 2 Spectrum of oleic acid



SEM analysis Biosynthesized ZnO nanoparticles

The morphology of Zn O NPs was spherical. The mean diameter of spherical NPs was 12.6 ± 3.8 nm [crystalline size less than 20 nm surrounded by the capping agent]. Combination therapy was the most potent therapy for plants. [Figure: 1]

Figure: 1 SEM Analysis



Isolation of Erwinia amylovora in Kings B media:

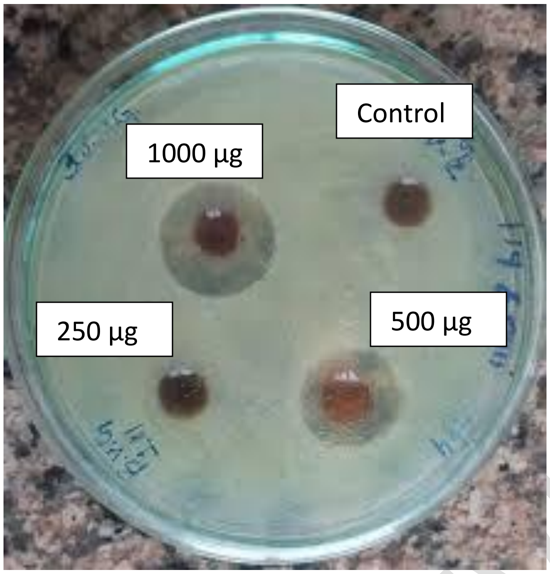
Colonies of *E. amylovora* on Kings media are pale violet, circular, high convex to domed, smooth and mucoid, and they grow more slowly than on King's B media. [Figure: 2]

Figure: 2 Colony morphology

Minimum bactericidal concentration and time–kill determinations

Using time–kill tests, Erwinia sps was decreased by 68 % within 2 hours in the presence of 1000 µg/mL micro ZnO. This was enhanced to 88 % ($p < 0.05$; independent t-tests for students). In the presence of 1000g/mL micro ZnO, these overgrown bacteria were reduced to zero in 4 hours. *Spirulina platensis* inhibited *Erwinia amylovora* bacterial multiplication by 50% (IC50) at a concentration of 500 µg/mL of extract. Against the plant pathogenic strain, there was a considerable inhibitory impact ($p<0.0001$). [Figure: 3]

Figure: 3– Well diffusion assay



Seed germination began after one day, with a percentage ranging from 28 under control conditions to 50% with 500 µg/mL ZnO-NP. Seed germination began on the first day as well, but with a low percentage (less than 20% in all the treatments except at 1000 µg/mL concentrations, After five days of harvesting, better results were noticed in 1000µg/mL

ZnO-NP treatments influenced both root and shoot lengths. Root and shoot elongation was stimulated by low-moderate levels of ZnO-NPs, reaching a height of 7.6 ± 0.8 and 6 ± 0.7 cm, respectively, with 12.5 µg/mL of ZnO-NPs, following which the roots steadily decreased at higher concentrations of 100 and 200 µg/mL to 4 ± 0.3 and 4 ± 0.45 cm, respectively.

There was no substantial difference in root lengths between low and moderate levels compared to control, but they decreased drastically at high levels and registered the shortest lengths among all treatments at 100 and 200 base fluid dilutions (2.8 ± 0.2 and 2 ± 0.33 cm, respectively).

Discussion:

Notably, as multi-drug resistance bacteria have evolved, ZnO NPs have emerged as promising antimicrobial agents. This is mostly owing to their enhanced ability to tackle a wide range of infections. Furthermore, zinc is recognised as a necessary trace element for the majority of biological functions in the animal's body. As a result, the use of ZnO NPs has been shown to greatly improve the health and productivity of plant pathogens. [11]

Phenols derived from Solid Phase Extraction As a result, this was considered the most measured bioactive component of Spirulina platensis. H1 NMR spectroscopy investigations revealed the presence of phenolic chemicals, as well as long-chain alcohol groups. In SEM investigation, the mean diameter of spherical Phenols-ZnONPs enclosed by the capping agent is less than 15 nm. Concentrations of 1000g/mL were 42 percent, 33 percent, 20 percent, and 18 percent in cells with time periods of 4, 8, 16, and 24 hours. Spirulina platensis inhibited Erwinia amylovora bacterial multiplication by 50% (IC50) at 500 g/mL extract concentration. Against the plant pathogen, a considerable inhibitory impact ($p<0.0001$) was observed.

In contrast to our study, Rajendran et al., 2021 revealed the biosynthesis of zinc oxide nanoparticles (ZnO NPs) from crude extracts and phytochemicals has recently received a lot of interest. [12] Green NP synthesis is a cost-effective, environmentally friendly, and promising alternative to chemical synthesis. This research concerns the manufacture of ZnO NPs utilising *Rubus fairholmianus* root extract (RE) as an effective reducing agent. The RE-ZnO NPs were found to be spherical in form with clusters (1–100 nm) according to SEM examination. The NPs' antibacterial efficacy against Staphylococcus aureus was evaluated using agar well diffusion, minimum inhibitory concentration, and bacterial growth assays. The phytochemicals of *R. fairholmianus* aid in the formation of stable ZnO NPs and demonstrate antibacterial activity.

This might be owing to ZnO NPs' damaging effect on cells and increased formation of highly reactive oxygen species (ROS) such as OH, H_2 O_2, and O_{22}^- which causes cell death. After attaching to the surface of the cell membrane, ZnO nanoparticles cause a disruption in the cell's respiration due to interactions with enzymes in the bacteria's respiration chains, as well as increased permeability through the bacterial cells, resulting in the loss of the cell's transport mechanism (Sharaf et al., 2021).[12] This might be because ZnO NPs have a better penetrating capacity proved by Kalaba MH et al., 2021. [13]

Sarcophyton trocheliophorum 1H NMR revealed three aromatic protons at 7.30, 7.07, and 6.58, corresponding to 1,2,4-trisubstituted aromatic residue.[14]

Low doses of ZnO-NPs aided seed germination and seedling growth in *Vicia faba*, while higher concentrations (1000 and 2000µg/mL) caused phytotoxicity. These findings are similar to those of Youssef and Elamawi (2020). [15] Furthermore, Studies discovered that ZnO-NPs improved rice seed germination, which is similar to our findings. [16,17] Sharaf (2021) investigated the effects of 20-nm ZnO-NPs at concentrations of 0, 10, 20, 30, and 40 mg/L on onion root length (Allium cepa L). [12]

Hence the present study attributes the mechanism behind the bactericidal activity of Combined nanoparticles. We prove that phenol compound disturbs the cellular membrane of pathogenic bacteria and nanoparticles conjugated with the bioactive compound act as reactive oxygen species, which destroy the genetic material of the pathogen. However the entire study resembles hypothetically, Tiwari et al., 2018 [18] explained the mechanism of anti-bacterial activity of zinc oxide nanoparticle against carbapenem-resistant Acinetobacter baumannii.

Conclusion:

This research work is the ongoing portion of the previously biosynthesized ZnO-NPs using aqueous extract of *Spirulina platensis*, a well-known plant for its medicinal importance. The presence of bioactive compound has been confirmed by H nmr spectroscopy analysis. SEM analysis determined morphology and vibrational modes, while apparent charge and steadiness were determined by DLS. The produced Phenolic capped ZnO-NPs have shown good antibacterial capabilities. Phenolic capped ZnO-NPs have shown an effective bactericidal activity at a higher concentration and showed >50% inhibition against plant pathogen *Erwinia amylovora*. Our findings suggest that the above-mentioned Phenolic capped ZnO-NPs might be used in agriculture due to their anti-bacterial properties. Because of the manner of action of ZnONPs and phytochemicals, synergy and additive behaviour of ZnONPs-phytocompounds arises. Because bacteria cannot acquire resistance to this consortium, this conjugation is always advantageous.

Recommendations:

Future research in this topic should be in the treatment of different diseases such as tumour causes and other inflammatory disorders in plants. More study on BAC capped ZnO-NPs is needed to investigate their biological properties in vitro and in vivo. Further experiments should be performed, including in vivo measurements, and side effects of ingesting this compound should be thoroughly investigate.

Limitations:

No toxicity assay were analysed in the present study with the use of BAC capped ZnONPs

Ethical approval: Not required.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Tiwari V, Mishra N, Gadani K, Solanki PS, Shah NA, Tiwari M. Mechanism of anti-bacterial activity of zinc oxide nanoparticle against carbapenem-resistant Acinetobacter baumannii. Frontiers in microbiology. 2018 Jun 6;9: 1218.
2. Yusof HM, Mohamad R, Zaidan UH. Microbial synthesis of zinc oxide nanoparticles and their potential application as an antimicrobial agent and a feed supplement in animal industry: a review. Journal of animal science and biotechnology. 2019 Dec;10(1):1-22.
3. Kapoor RT, Salvadori MR, Rafatullah M, Siddiqui MR, Khan MA, Alshareef SA. Exploration of Microbial Factories for Synthesis of Nanoparticles–A Sustainable Approach for Bioremediation of Environmental Contaminants. Frontiers in Microbiology. 2021 Jun 4;12:1404.
4. Saied E, Eid AM, Hassan SE, Salem SS, Radwan AA, Halawa M, Saleh FM, Saad HA, Saied EM, Fouda A. The Catalytic Activity of Biosynthesized Magnesium Oxide Nanoparticles (MgO-NPs) for Inhibiting the Growth of Pathogenic Microbes, Tanning Effluent Treatment, and Chromium Ion Removal. Catalysts. 2021 Jul;11(7):821.
5. Munirathna, K.S.P., Coswatte, A.C.W.W.M.C.L.K. and Jayamanne, S.C., 2019. Development of Low Cost Mass Culture Media for Spirulina platensis. DOI: 10.31364/SCIRJ/v9.i03.2021.P0321844

6. Mencin, M.; Mikulic-Petkovsek, M.; Veberiˇc, R.; Terpinc, P. Development and Optimisation of Solid-Phase Extraction of Extractable and Bound Phenolic Acids in Spelt (Triticum spelta L.) Seeds. Antioxidants 2021, 10, 1085. https://doi.org/10.3390/antiox10071085
7. Lambert JB, Santiago-Blay JA, Wu Y, Contreras TA, Johnson CL, Bisulca CM. Characterization of Phenolic Plant Exudates by Nuclear Magnetic Resonance Spectroscopy. J Nat Prod. 2021 Sep 24;84(9):2511-2524. doi: 10.1021/acs.jnatprod.1c00522. Epub 2021 Sep 7. PMID: 34491068.
8. Adhikari S, Adhikari A, Ghosh S, Roy D, Azahar I, Basuli D, Hossain Z. Assessment of ZnO-NPs toxicity in maize: An integrative microRNAomic approach. Chemosphere. 2020 Jun 1;249:126197.
9. Upadhyaya, H.; Roy, H.; Shome, S.; Tewari, S.; Bhattacharya, M.; Panda, S.K. Physiological Impact of Zinc Nanoparticle on Germination of Rice (Oryza Sativa L) Seed. J. Plant Sci. Phytopathol. 2017, 1, 062–070.
10. Shaaban, M., Ghani, M.A. and Issa, M.Y., New Naturally Occurring Compounds from Sarcophyton trocheliophorum, 2021, 12 (2): 2285 – 2331. https://doi.org/10.33263/BRIAC122.22852331
11. Rajendran, N.K.; George, B.P.; Houreld, N.N.; Abrahamse, H. Synthesis of Zinc Oxide Nanoparticles Using Rubus fairholmianus Root Extract and Their Activity against Pathogenic Bacteria. Molecules 2021, 26, 3029. https://doi.org/10.3390/molecules26103029
12. Sharaf, M.H.; El-Sherbiny, G.M.; Moghannem, S.A.; Abdelmonem, M.; Elsehemy, I.A.; Metwaly, A.M.; Kalaba, M.H. New Combination Approaches to Combat Methicillin-Resistant Staphylococcus Aureus (MRSA). Sci. Rep. 2021, 11, 1–16.
13. Kalaba MH, Moghannem SA, El-Hawary AS, Radwan AA, Sharaf MH, Shaban AS. Green Synthesized ZnO Nanoparticles Mediated by Streptomyces plicatus: Characterizations, Antimicrobial and Nematicidal Activities and Cytogenetic Effects. Plants. 2021 Sep;10(9):1760.
14. Shaaban, M., Ghani, M.A. and Issa, M.Y. New Naturally Occurring Compounds from Sarcophyton trocheliophorum. 2021 12 (2): 2285 – 2331

15. Youssef MS, Elamawi RM. Evaluation of phytotoxicity, cytotoxicity, and genotoxicity of ZnO nanoparticles in Vicia faba. Environmental Science and Pollution Research. 2020 Jun;27(16):18972-84.
16. Agarwal H, Menon S, Kumar SV, Rajeshkumar S. Mechanistic study on antibacterial action of zinc oxide nanoparticles synthesized using green route. Chemico-biological interactions. 2018 25;286:60-70.
17. Gupta M, Tomar RS, Kaushik S, Mishra RK, Sharma D. Effective antimicrobial activity of green ZnO nano particles of Catharanthus roseus. Frontiers in microbiology. 2018 Sep 3;9: 20-30.
18. Tiwari V, Mishra N, Gadani K, Solanki PS, Shah NA, Tiwari M. Mechanism of anti-bacterial activity of zinc oxide nanoparticle against carbapenem-resistant Acinetobacter baumannii. Frontiers in microbiology. 2018 Jun 6;9: 1218.