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Research Article

Antioxidant, Antimicrobial Activity, and Cytotoxic Effects on Liver Cancer Cell Lines of SnO2/Calcite Bio-NanoComposite from Cypress (*Cupressus sempervirense L.*) and Eggshell Wastes

Atena Naeimi[1*], Fereshteh Ezzati Ghadi[2*], Seyed Mehdi Saadatkhah[3], Moones Honarmand[3]

[1]Department of Chemistry, University of Jiroft, Jiroft, Iran;[2]Department of Biology, University of Jiroft, Jiroft, Iran;[3]Department of Chemical Engineering, Birjand University of Technology, Birjand, Iran

ABSTRACT

Green fabrication of SnO_2/Calcite bio-nano composite was synthesized based on cypress leaves and eggshell wastes. SEM, TEM, XRD, EDAX, elemental analysis, and FT-IR was used for more information about this composite. Free radical scavenging, total antioxidant capacity, antimicrobial, and cytotoxicity of this bio-nano composite investigated. SnO_2/Calcite bio-nano composite was displayed antioxidant and antimicrobial properties successfully. Cytotoxic potential of SnO_2/Calcite bio-nano composite against HepG2 cells observed. The results suggest that SnO_2/Calcite bio-nano composite holds more significant potential than calcite against bacteria and fungi.

Keywords: Bio-nano composite; Anticancer; Antioxidant; Antimicrobial

INTRODUCTION

Chemotherapy and surgery are the most common approach for liver cancer therapy [1-4], and paclitaxel [5], Adriamycin [6], and cisplatin injection [7] are its essential drugs [8]. In this regard, the most potential advantage of new technologies is using composite materials in cancer treatment [9]. Hence, advances in green nanocomposites can create a new view in the treatment of cancer and its challenges. Also, their physicochemical properties, high surface-to-volume ratio, controlled size, and the electron's spatial confinement, shape, and morphology can help to have the extension used in this field [10-13]. Calcium carbonate (calcite) has widespread interest in pharmaceutical sciences due to its biocompatibility. For the synthesis of anticancer medicines, pH sensitivity and functionalization utilizing target agents are of particular interest. It seems that the slow degradation of calcite nanoparticles is essential to have high drug retention times in cancer tissues [14,15]. SnO_2, an n-type semiconductor, has with wide band gap (Eg: 3.6–3.8 eV), and it is an excellent model to study the effect of surface excess on nanocomposites. Due to strengthen transparency in the visible region, high thermal stability, chemically inert, mechanically hard, and low operating temperature, it has various biomedical applications [16-18]. SnO_2 NPs have shown excellent antimicrobial, anticancer, and antioxidant activity [19,20]. Cytotoxicity of SnO_2 on hepatocellular carcinoma (HepG2) reported [21,22]. *Cupressus sempervirens L.* (Mediterranean cypress) is an evergreen tree belonging to the Cupressaceae family. Cypress is native to North America, the Mediterranean region, and Iran. It is recognized as a medicinal herb and is applied as a diuretic, digestive stimulant, cold-fighting agent, and wound healer. The leaves of this plant are rich in flavonoids and tannins [23,24]. In this study, SnO_2/Calcite as a novel bio-nano composite was engineered based on eggshell wastes and cypress leaves. For the first time, cytotoxic effects of one bio-nano composite based on natural materials evaluated on HepG2 liver cancer cell lines. Also, antioxidant, antimicrobial activities of SnO_2/Calcite nano biocomposite were considered.

Correspondence to: Atena Naeimi, Department of Chemistry, University of Jiroft, Jiroft, Iran, Tel: 7867161167;Email: a.naeimi@ujiroft.ac.ir Fereshteh Ezzati Ghadi, Department of Biology, University of Jiroft, Jiroft, Iran; Email: f.ezzati@ujiroft.ac.ir

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MATERIALS AND METHODS

The tin (II) chloride dehydrate ($SnCl_2.2H_2O$) was purchased from Sigma-Aldrich company. Plant collected from South Khorasan (Iran) and eggs purchased from the local supermarket. SnO_2/Calcite bio-nano composites and eggshell evaluated by X-ray Diffraction (XRD) on a Philips model PW 1800 to consider its purity. The shape and particle size distribution of the SnO_2/Calcite bio-nano composites were specified TEM (ZEISS EM900). Energy-dispersive X-ray spectroscopy (EDS- ZEISS, EVO18) and scanning electron microscopy (SEM-ZEISS, EVO18) analyses carried out to study the chemical composition and morphology of SnO_2/Calcite bio-nano composites.

Plant collection and preparation of the aqueous extract cypress leaves

The leaves of cypress were collected from South Khorasan, Iran, and authenticated by H. Bibak, Faculty member of the Department of Biology, University of Jiroft. A voucher specimen with accession number 203 deposited to the Herbarium of Department of Biology, Faculty of Science, University of Jiroft. The cypress leaves washed with distilled water to remove dust particles and dried in the shade for two weeks. The dried leaves were powdered into fine particles using a mechanical grinder. The dried powder of cypress leaves (10 g) refluxed using distilled water (100 mL) for 30 minutes. The Cypress extraction solution was used for the green synthesis of nanomaterials in the next steps after filtration.

Biosynthesis of SnO_2 nanoparticles

25 mL of $SnCl_2.4H_2O$ (0.05M) was added to 50 mL of extraction solution and stirred at 25°C for 30 minutes. After cooling, the synthesized precipitates were separated using a centrifuge and washed with water, and dried. Finally, they were placed in an electric furnace at 550°C for one hr.

Biosynthesis of SnO_2/Calcite bio-nano composites

The chicken eggs were braked and separated from the adhering membrane inside them. After washing the eggshells with distilled water and drying at 100 °C for two h, they powdered using a mortar. After mixing the eggshell powder (1 g) and CH_2Cl_2 (5 mL) within 10 minutes, the eggshell powder filtered and dried for the next step. In the following, the tin (II) chloride dihydrate solution (50 mL, 0.05 M) and dried eggshell (1 g) mixed within 30 minutes, and the extract of the cypress leaves (100 mL) added drop by drop within 30 minutes and refluxed for another 30 minutes. Then, the synthesized precipitates were centrifuged at 15000 rpm and washed with water, and dried. The biosynthesized nanocomposites were placed in an electric furnace at 550°C for one hr.

Free radical scavenging activity

3 mL of various concentrations of pure eggshell and SnO_2/Calcite (50, 100 and 200 µg/mL) was treated with 1 mL of 0.1 mM DPPH (1,1 diphenyl-2-picrylhydrazyl) reagent (in 96% ethanol) [25]. After incubating within 30 min at 37 °C, the absorbance considered at 517 nm using spectrpphotometer. Ascorbic acid (dissolved in 96% ethanol) and 96% ethanol were used as standard and blank, respectively. The percentage of radical scavenging activity was measured utilizing the equation:

% scavenging activity= (1-As / Ac) × 100

Where;The absorbance of the DPPH solution is As. The absorbance of control is Ac using reagent instead of sample.

Total antooxidant capacity

900 µL of the mixture of 0.6 M sulfuric acid, 28 mM sodium phosphate, and four mM ammonium molybdate reacted with a 100 µL (dissolved in 0.1% DMSO) aliquot of each sample. It heated for 90 min at 95 °C in the water bath. Absorbance considering at 630 nm after diminishing the temperature, and ascorbic acid used as a positive control. The number of µg equivalents of ascorbic acid per milligram sample used for total antioxidant capacity (i.e., µgAAE/mg sample) [26].

Evaluation of cytotoxicity of SnO_2/Calcite bio-nano composite

Standard MTT assay was used to determining the cell viability, and cytotoxicity of this bio-nano composite [22]. For MTT assay, samples were prepared by dissolving in 0.1% DMSO. Hepatocellular carcinoma (HepG2) cell lines were provided from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). The mixture of Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), two mM L-glutamine, 100 mg/L streptomycins, and 100 IU/mL penicillin used for growing the cells. Then were maintained at 37°C in a 5% carbon dioxide humidified incubator. The fully grown cells were harvested and seeded in 96-well plates at a concentration of 1 × 105 cells/well. Cells were cleaned with 100 µL of serum (free medium) two times after 24 h incubation and starved for 1 hour at 37°C. Cells incubated in the presence of different concentrations of SnO_2/calcite (10, 50, 100, 250, and 500 µg/mL) for 72 h at 37 °C, after starvation. After aspiration, serum-free medium containing 0.5 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide

(MTT) was added and incubated for four h at 37 °C in a CO_2 incubator. Then the cells were washed with 200 µL of Phosphate-Buffered Saline (PBS). The crystals dissolved by adding 100 µL of 0.1 % Di-Methyl SulfOxide (DMSO) and optical density determined at 570 nm for each well-containing cell.

Antimicrobial activities by disk diffusion method

Antimicrobial properties of the bio-nano composite against Lactobacillus sakei subsp. Sakei (Gram-positive, ATCC 15521), *Escherichia coli* (Gram-negative, ATCC 25922), and Saccharomyces cerevisiae (ATCC 9763) investigated using the standard disk diffusion technique.Briefly, bacteria strain and fungi were sub-cultured overnight in a nutrient broth medium. 50 µL of the bio-nano composite and pure eggshell at different concentrations (50, 100, and 200 µg/disc) was added to the paper disc (6.4 mdiameter) and dried for 10 min. Pure eggshell and SnO_2/Calcite bio-nano composite dissolved in 5% Dimethyl

Sulfoxide (DMSO). An overnight strain (1× 107 CFU/mL) was evenly spread on nutrient agar plates with a sterile swab and allowed to dry for 15 min, and then, plates were incubated 24 h at 37°C. Gentamycin (50, 100, and 200µg/disc) was used as a positive control [27]. (11%)

Statistical analysis

All the biological assays have done in triplicate, and the results presented as Mean ± Standard Deviation (SD). Statistical significance of groups measured by one-way ANOVA followed using an LSD post hoc test. The significance was presented as a P value less than 0.05.

RESULTS AND DISCUSSION

In this work, for the first time, the biosynthesis of SnO_2/Calcite bio-nano composite was carried out using an aqueous extract of cypress leaves. When the 20 g of leaves has used, the magnet inside the round-bottomed flask did not stir well due to a large number of leaves whereas, when the leaves amount was 5 g, the synthesis of SnO_2/Calcite bio-nano composite was low. So, the amount of 10 g of leaves showed as the optimal value. So, SnO_2/Calcite nanocomposite produced by calcination of sample from a mixture of tin salt and eggshell at the presence of cypress leaves. Cypress leaves extract act as an excellent reducing agent and green media for the biosynthesis of SnO_2/Calcite bio-nano composite. The crystalline structure of $CaCO_3$ and this bio-nano composite were considered using XRD.

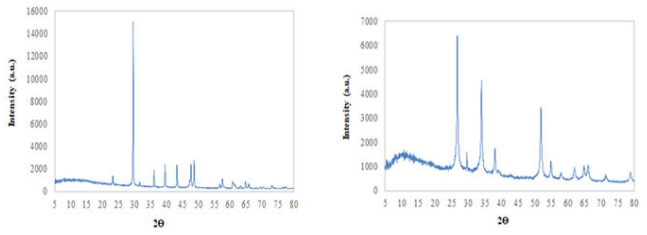

Figure 1: XRD of (a) pure eggshell and (b) SnO_2/Calcite bio-nano composites.

Figure 1 (a) and (b) shows the XRD patterns of pure eggshell and SnO_2/Calcite bio-nano composite, respectively. For the pure eggshell, found that the angles 2θ=23.0°, 29.4°, 31.4°, 35.2°, 39.4°, 43.1°, 47.5°, 48.5°, 56.6°, 57.4°, and 60.6°attributed to the typical diffraction peak of rhombohedral $CaCO_3$ [25-29]. In comparison with pure eggshell, several different diffraction peaks at 2θ = 26.61°, 33.89°, 37.95°, 51.78°, 54.75°, 61.87°, and 64.71° indexed to (110), (101), (200), (211), (220), (310), and (112) Bragg's reflections of SnO_2 nanoparticles [30]. In addition, no other diffraction peaks are observed, revealing the pure phase of SnO_2 and $CaCO_3$. Analyzing these characteristic peaks by taking pure eggshell shows an absorption peak centered at 3494 cm−1, generally recognized as the stretching vibration of the −OH in the adsorption water (Figure 2a) [31]. The three weak absorption peaks at 2976, 2875, and 1801 cm−1 could determine by the presence of C=O bonds from carbonates [29]. The peak at 2517 cm−1 could attribute to the characteristic peaks of CaCO3 [32].

The most substantial absorption peak at 1422 cm−1 could consider as the C=O stretching modes of $CaCO_3$ [29]. The peak at 876 and 713 cm^{-1} correspond to internal and external deformation mode unique
to $CaCO_3$ [16]. The absorption peak at position 1635 cm^{-1} can be regarded as the vibration of the N-H bond of a specific type of protein [13]. Compared to pure eggshell, the infrared signature of all catalysts has two more absorption peaks, located at positions of 586 and 669 cm^{-1}, respectively [33]. Furthermore, the absorption peak of metal oxidation situated in this range; hence these two absorption peaks are considered to
belong to the absorption [34]. The loading of the SnO_2 nanoparticles does not affect on the absorption peaks of these functional groups [35] (Figure 2b).

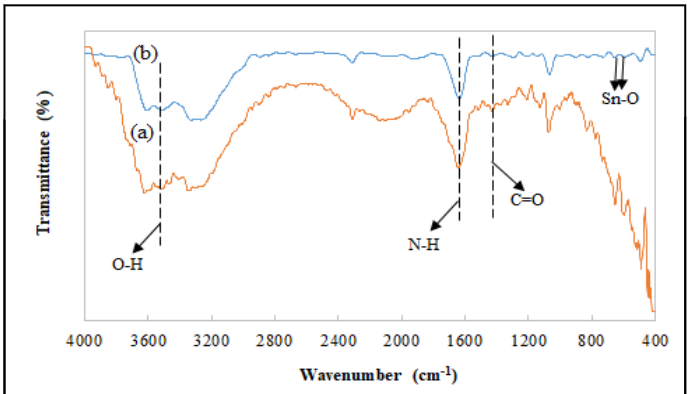

Figure 2: FT-IR of pure eggshell (a) SnO_2/Calcite bio-nano composite (b) SnO_2 nanoparticles does not affecting the absorption peaks of the functional groups.

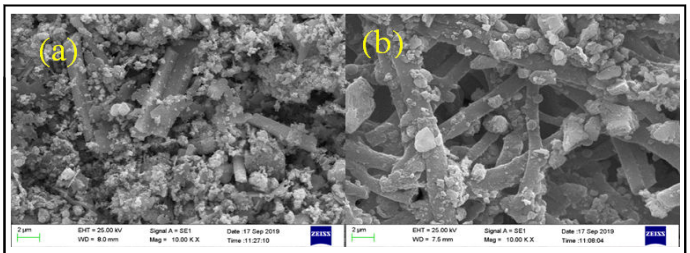

Figure 3: SEM images of (a) eggshell pure and (b) SnO2/ Calcite bio-nano composites.

SEM images of pure eggshell (Figure 3a) and SnO_2/Calcite bio-nano composite (Figure 3b) shown their morphology and size of particles. Figure 3a shows a high-magnification micrograph of the fibrils building the eggshell pure. After adding the tin salt and cypress leaves and formation SnO_2/Calcite bio-nano composite, the fibril wet were broken, and there are many SnO_2 nanoparticles in it (Figure 3b). The obtained results encourage us to have more information about this novel bio-nanocomposite. The TEM image, EDAX, and elemental analysis show the size of nanoparticles, presented elementals, and their percentages (Figure 4a and 4b). The average length of nanoparticles is 25 nm according to the TEM image (Figure 4a). Carbon, calcium, tin, and oxygen are the elementals of it (Figure 4b). The percentage of C, Ca, Sn, and O are 11.63, 2.17, 14.50, and 71.69% (Figure 4b).

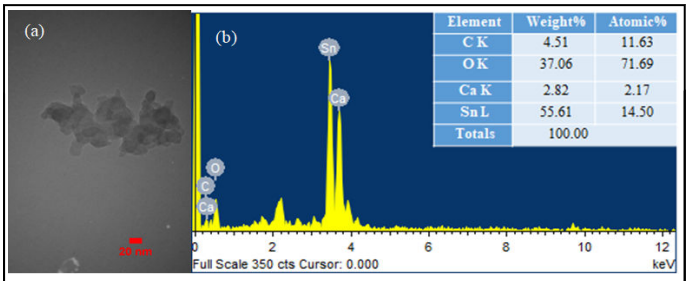

Figure 4: TEM image: (a) EDAX and (b) elemental analysis of SnO_2/Calcite bio-nano composites.

Antioxidant properties

Antioxidant potential of the synthesized SnO_2/Calcite bio-nano composite and pure eggshell investigated using DPPH radical scavenging assay. The percentage of free radicals scavenging and antioxidant activity was confirmed by turning the purple solution containing DPPH to yellow (Figure 5).

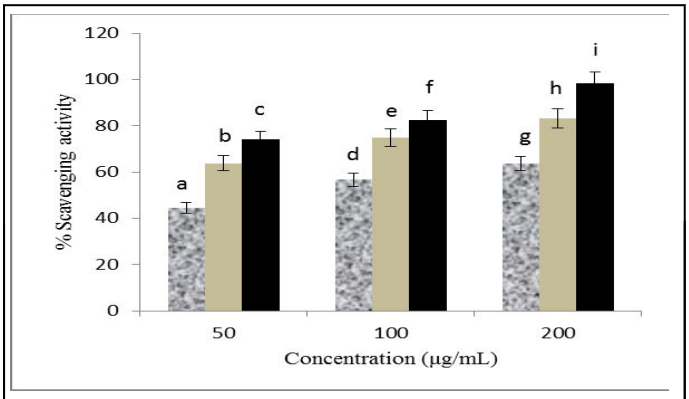

Figure 5: Percentage scavenging activity of pure eggshell andSnO_2/Calcite bio-nano composite. Values present as Mean ± SD from three independent experiments. Different letters denoted a significant difference with $p<0.05$. The same letters indicated no significant differences between groups.
Note: ( ) Pure Eggshell, ( )SnO_2/Calcite, ( ) Vit C.

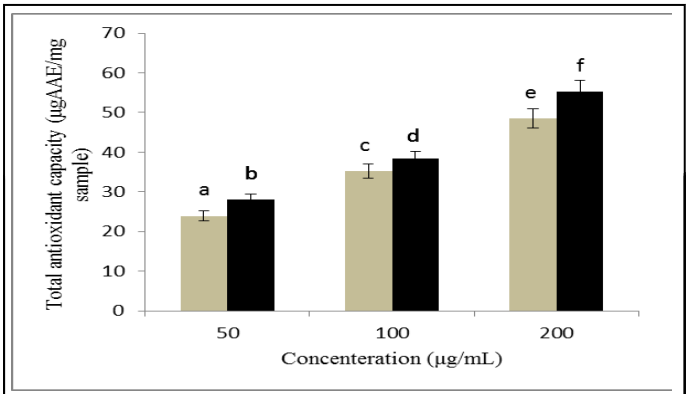

Figure 6: Total antioxidant capacity of pure eggshell and SnO_2/Calcite. Values are mean ± SD from three independent experiments. The different letters indicated significant differences between groups.
Note: ( )Pure Eggshell, ( )SnO_2/Calcite.

Pure eggshell percentage in 50, 100 and 200 µg/mL showed 44.51 ± 0.05, 56.64 ± 2.09 and 63.61 ± 1.23, and SnO_2/Calcite showed 63.80 ± 1.63, 74.77 ± 1.58 and 83/29 ± 0.64, respectively. The obtained results showed that the highest concentration showed maximum antioxidant activity [36]. By this study, the antioxidant activity of SnO_2 nanoparticles reports through DPPH assay [37].

Results showed that SnO_2/Calcite ($p<0.001$) indicated higher antioxidant activity than pure eggshell for various concentrations. The reduced DPPH gains by pairing the DPPH radical electron with a cation from a free radical scavenging antioxidant. Also, the total antioxidant capacities of SnO_2/Calcite and pure eggshell showed in Figure 6. Changing Mo (VI) to Mo (V) through antioxidant mediators corresponds to the total antioxidant capacity, and green color is related to the formation of phosphate/Mo (V) complex, and absorption at 695 nm observed. It shows that pure eggshell nanoparticle has lower total antioxidant activity than SnO_2/Calcite bio-nano composite. Antibacterial and antifungal potential of synthetized eggshell nanoparticle and SnO_2/Calcite bio-nano composite assessed in terms of zone of inhibition of microbial growth (Figures 7-13).

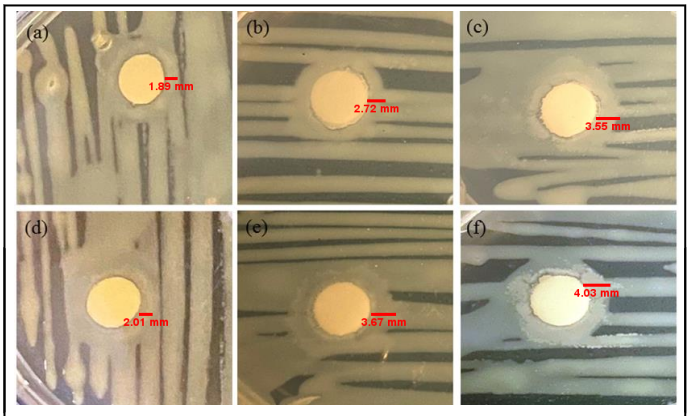

Figure 7: The antibacterial activity of pure eggshell and SnO_2/Calcite against *E.coli* bacteria. (a): the inhibition zone of 50 µg/disc pure eggshells, (b): the inhibition zone of 100 µg/disc pure eggshells, (c): the inhibition zone of 200 µg/disc pure eggshells,(d): the inhibition zone of 50 µg/disc SnO_2/Calcite, (e): the inhibition zone of 100 µg/disc SnO_2/Calcite and (f): the inhibition zone of 200 µg/disc SnO_2/Calcite.

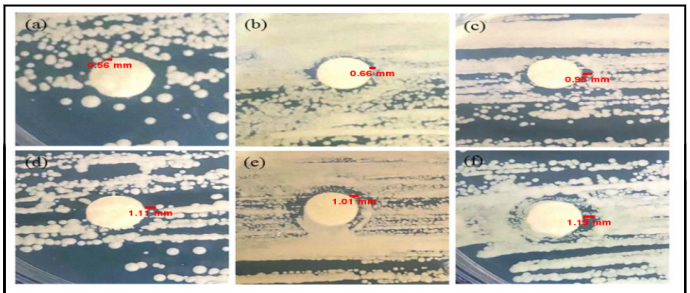

Figure 8: Antifungal activity of pure eggshell and SnO_2/Calcite against Saccharomyces cerevisiae. (a): the inhibition zone of 50 µg/disc pure eggshells, (b): the inhibition zone of 100 µg/disc pure eggshells, (c): the inhibition zone of 200 µg/disc pure eggshells, (d): the inhibition zone of 50 µg/disc SnO_2/Calcite,(e): the inhibition zone of 100 µg/disc SnO_2/Calcite and (f): the inhibition zone of 200 µg/disc SnO_2/Calcite.

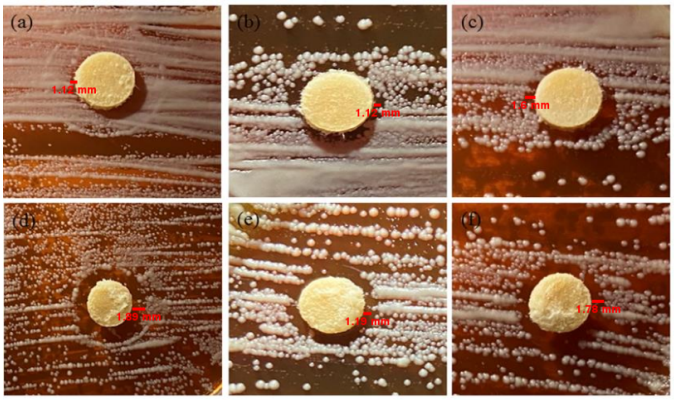

Figure 9: Antibacterial activity of pure eggshell and SnO_2/Calcite against Lactobacillus sakei subsp. Sakei. (a): the inhibition zone of 50 µg/disc pure eggshells, (b): the inhibition zone of 100 µg/disc pure eggshells, (c): the inhibition zone of 200 µg/disc pure eggshells, (d): the inhibition zone of 50 µg/disc SnO_2/Calcite, (e): the inhibition zone of 100 µg/disc SnO_2/Calcite and (f): the inhibition zone of 200 µg/disc SnO_2/Calcite.

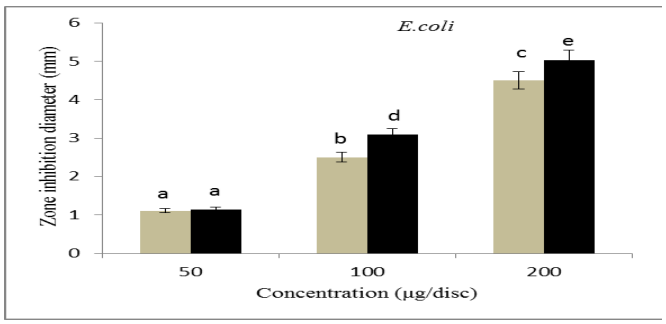

Figure 10: Zone of inhibition of pure eggshell and SnO_2/Calcite against *E.coli*. Values are mean ± SD from three independent experiments. The different letters indicated significant differences ($p<0.05$) between groups.
Note: ( ) Pure Eggshell, (■) SnO_2/Calcite.

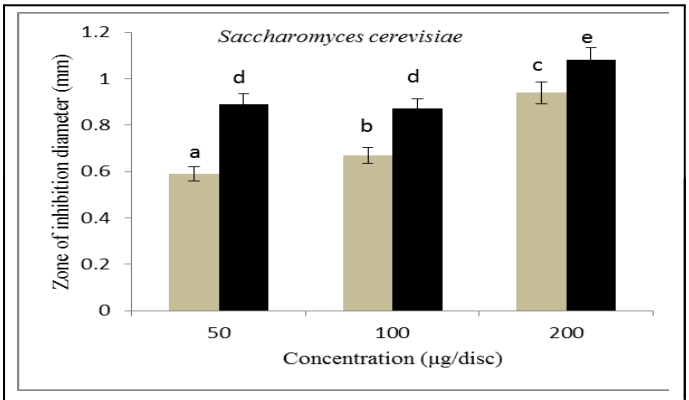

Figure 11: Zone of inhibition of pure eggshell and SnO_2/Calcite against Saccharomyces cerevisiae. Values are mean ± SDfrom three independent experiments. The different letters indicated significant differences ($p<0.05$) between groups.
Note: ( ) Pure Eggshell, (■) SnO_2/Calcite.

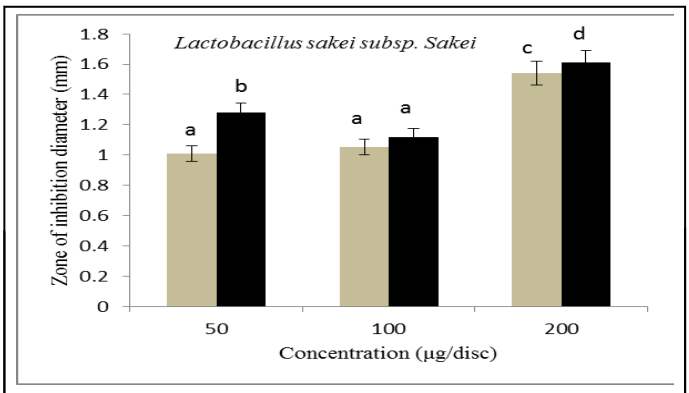

Figure 12: Zone of inhibition of pure eggshell and SnO_2/Calcite against Lactobacillus sakei subsp. Sakei. Values are mean± SD from three independent experiments. The different letters indicated significant differences ($p<0.05$) between groups.

Note: ( ) Pure Eggshell, (■) SnO_2/Calcite.

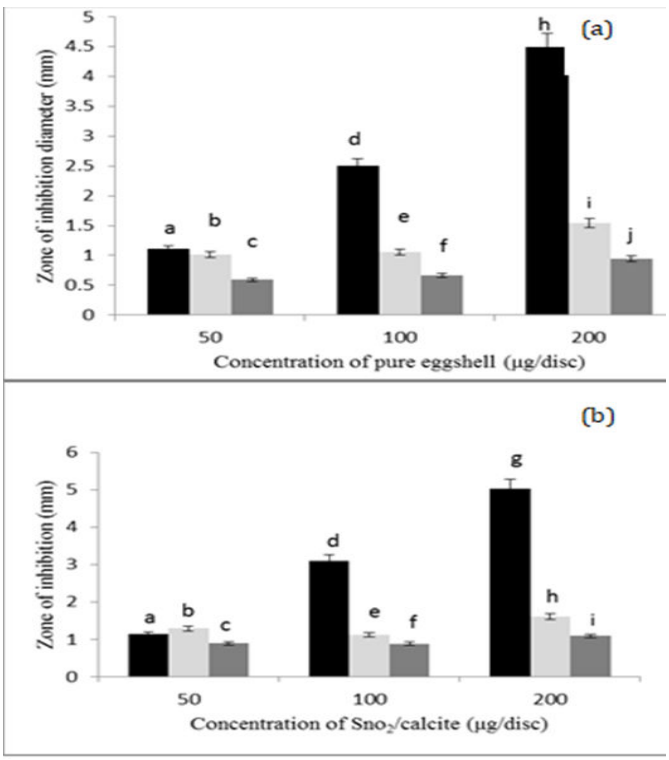

Figure 13: Zone of inhibition of pure eggshell and SnO_2/Calcite against Lactobacillus sakei subsp. Sakei. Values are mean± SD from three independent experiments. The different letters indicated significant differences ($p<0.05$) between groups.

Note: (■) *E.coli*, ( ) *L.sakie*, ( ) *S.cervisiae*.

SnO_2/Calcite bio-nano composite (200 µg/disc) showed the highest antibacterial activity against Gram-positive and Gram-negative bacteria (5.03 ± 0.01 and 1.61 ± 0.02 mm). Results from Figure 8 and 11 showed that the diameter of inhibition zone of fungi strains in bio-nano composite was 1.08 ± 0.02 mm,

whereas in the pure eggshell was 0.94 ± 0.03 mm, respectively. The antimicrobial results showed that the bio-nano composite of SnO_2/Calcite was more effective than pure eggshell.

Antibacterial activity

The results obtained from Figure 13 showed that all concentrations had antimicrobial activity for all bacteria evaluated. The highest antibacterial activities were against *E.coli* in SnO_2/Calcite (200 μg/disc) bio-nano composite. Lower antibacterial activities, which were also considered adequate, were against Saccharomyces cerevisiae (50μg/disc) in pure eggshell. Metal oxide nanoparticles tend to absorb on the cell membrane of bacteria. The antibacterial properties of SnO_2 nanoparticles have been attracting attention due to the generation of active oxygen species which interact with the cell membrane of the bacteria and penetrate through it [38]. The difference of structural and chemical composition on cell wall bacteria may be responsible for its zone of inhibition [39].

It is a fact that eggshell nanoparticles possess good antibacterial activity [40]. The antimicrobial activity of SnO_2 nanoparticles and eggshell can be due to breaking the cell wall membrane and thereby causing the disturbance in the cell function [41-43]. Wang et al. reported that CaCO3 found to behave as a physical barrier to protect the body in the natural environment from external mechanisms and microbial damage [44]. Therefore, Sno2/calcite biocomposite showed higher antibacterial activity than calcite nanoparticles due to more metal oxide contained.

Cytotoxicity (MTT) assay

Cell cytotoxicity studies of SnO_2/Calcite bio-nano composite evaluated using MTT assay. Figure 14 shows the percentage cell viability of the HepG2 cells. The cell viability decreased as the increased concentrations of SnO_2/Calcite. After 500 μg/mL SnO_2/Calcite bio-nano composite treatment, the percentage of cell viability compared with untreated cells decreased to 58%, whereas, after treatments of the cells with concentrations of 50, 100, and 250 μg/mL of bio-nano composite, the percentage of cell viability was 85.6%, 86%, and 85.7%. SnO_2/Calcite bio-nano composite with a concentration of 10 μg/mL showed 102% cell viability. Results obtained from Figure 14 showed that SnO_2/Calcite bio-nano composite with the highest concentrations (500 μg/mL) had the most significant cytotoxic effect on HepG2 cell lines than other concentrations. These results indicated that bio-nano composite had significant cytotoxicity against Hepatocellular Carcinoma Cell Line (HepG2). It seems that cell tested, the approach of synthesis, and polymer using in the bio-nano composite are vital rules on the cytotoxic activity of them [45].

Biosynthesized SnO_2 nanoparticles show cytotoxic responses against HepG2. It seems that cytotoxicity of SnO_2 nanoparticles on HepG2 enhance by increasing the nanoparticle concentration 22. The decreased pH found in the extracellular environment of cancer cells uses for its detection and treatments. The pH-sensitive $CaCO_3$ nanoparticles can be used for cancerous diseases and unload its antitumor drug in a controlled release way because nanoparticles slow biodegradability [46].

CONCLUSION

The SnO_2/Calcite bio-nano composite synthesized using egg wastes and cypress leaves. The average size of this nanocomposite is 25 nm, according to the TEM image. SEM image shows that the fibril shape building of eggshell broken in the nanocomposite. EDAX data show carbon, calcium, tin, and oxygen as the presented elements in this composite. Cytotoxic potential of SnO_2/Calcite bio-nano composite against HepG2 cells evaluated. Excellent antioxidant and antimicrobial activities of this bio-nano composite observed. SnO_2/Calcite nano-biocomposite could have potential roles in future therapeutic applications due to bio-accessibility, bio-availability, and economically affordable. So, it could replace many treatment methods in diseases such as cancer and microbial cancer infections.

DATA AVAILABILITY

All data generated for this study included in the article.

ACKNOWLEDGMENT

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DECLARATIONS

Not applicable

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