

Phytochemicals, Biodegradation, Cytocompatibility and Wound Healing Profiles of Chitosan Film embedded Green Synthesized Antibacterial ZnO/CuO Nanocomposite

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Abstract

Open wound ulcer treatment remains a great challenge in wound care management especially involving elderly patients and multidrug resistance pathogen. Green-synthesized antibacterial nanocomposites have shown promising benefits in treating open wound ulcers. The present work studied the phytochemicals, biodegradation, storage, cytocompatibility and wound healing profiles of chitosan (Cs) film embedded with ZnO/CuO nanocomposites synthesized from of *Calotropis gigantea* (*C. gigantea*) leaf extract. HPLC was used to detect Quercetin bioactive constituent. Our cytocompatibility study demonstrated ZnO/CuO-Cs-1wt.% nanocomposites displayed highest cell viability (~ 169%) at 72 hours treatment. The biodegradation rate of ZnO/CuO-Cs-1wt.% increased proportionally with days of immersion and fully dissolved in 8 days while, commercial control degraded partially. The ZnO/CuO-Cs-1wt.% also exhibited significantly enhanced cell migration (26.81 $\mu\text{m}/\text{h}$) and wound closure (62.35 $\pm$ 9.46%) at 12 hours. This finding is also supported by our *in vivo* excisional open wound studies in *Sprague-Dawley* (*SD*) rats, which showed progressive recovery in 14 days. The controllable release of multiple metal ions (i. e., Zn^{2+} , Cu^{2+} and Ca^{2+}), reactive oxygen species (ROS), and bioactive elements from green synthesized ZnO/CuO-Cs nanocomposites might contribute to the wound recovery proses. This study highlighted the promising outcomes exhibited from green synthesized ZnO/CuO-Cs-1wt.% in wound healing technology.

1. Introduction

The biological healing response of open wound ulcers is interrupted and delayed by many local and systemic factors such as medication, obesity, smoking and alcoholism, microbial infection, age of bedridden and long hospitalized patients, sex hormones, stress level, history of serious illness like diabetes and oxygenation [1, 2]. Presently numerous scientific studies have yielded fabrication of an ideal wound dressing materials with biodegradable properties and a detailed *in vivo* toxicological investigation using animal model (i. e., rats, mice and rabbits) in tackling deficiencies in chronic wound healing. The reason hybrid albino male *Sprague-Dawley* (*SD*) rats were mostly chosen by scientists because they have calm temperament, easy handling, hormonal aspect and been widely used in safety test in the biomedicine [3, 4, 5]. Currently, polymer-based wound dressing material attracts countless attention in wound healing application (Table 1.0).

Microbiologically-mediated biodegradation is another important aspect in producing value-added wound dressing materials. Currently, non-biodegradable synthetic polymers create more plastic waste and it became biggest environmental threats and contaminants. This issue driving force amongst scientist in formulating new biodegradable chitosan (Cs) based biomaterials with enhanced structural properties. This set of experiment was carried out to investigate and compare wound healing performance of optimized Cs nanocomposites (ZnO/CuO-Cs-1wt.%) using an *in vitro* wound scratch method and *in vivo* excisional open wound in adult male *SD* rats. The expected outcome of this study to have potential biodegradable and biocompatible wound dressing materials which encourage faster wound healing; enhance cell proliferation; bring complete re-epithelialization; giving miraculous scar reduction and effective in treating open wound ulcers.

Table 1.0

An ideal wound dressing material with biodegradable properties and *in vivo* animal toxicological investigation.

Wound dressing	Animal	Size of created wound	Wound closure rate (%)	Total days for full recovery (day)	Wound stimulation mechanism	Biodegradation rate	Refs
AMP- and CeON-loaded catechol-modified hydrogel	SD rats	Ø= 8.0 mm	100	14	ROS (1–10 nmol H ₂ O ₂)	100% in 14 days	[6]
Oligomer Chitosan/Polyvinylpyrrolidone	Male albino mice	Ø= 10 mm	100	10	Electron-rich oxygen atoms	Nil	[7]
Curcumin grafted hyaluronic acid modified pullulan polymers	Wistar rats	Ø= 1.5 cm	100	21	Curcumin and ROS	Nil	[8]
Polygalacturonic/Hyaluronic acid embedded silver nanoparticles	Male rats	Ø= 2.5 cm	100	14	Nil	Nil	[9]
Bilayered multi-functional electrospun nanofibrous membrane - quaternized silicone and quaternized chitosan	Rabbits	Ø= 7 mm	97	14	Nil	Nil	[10]
Gold/perlite nanocomposite using <i>Urtica dioica</i> extract and its chitosan-capped derivative	BALB/c male mice	7 mm ²	100	14	Antioxidant	Nil	[11]
PVP/PLA-PEO complex nanofibers encapsulating collagen and cefazolin	Albino Swiss-Webster mice	Ø= 10 mm	100	14	Nil	Nil	[12]
Chitosan/Sodium Alginate/Velvet Antler Blood Peptides Hydrogel	Male ICR mice	Ø= 1 cm	100	14	Numerous short-chain peptides in VBPs	59.26% in 14 days	[13]
Chitosan/PDLLA-PEG-PDLLA solution	Male SD rats	Ø= 1 cm	95	7	Complex hydrophilic polymers with a high-water content	No degradation	[14]

Table 1.0

An ideal wound dressing material with biodegradable properties and *in vivo* animal toxicological investigation (continued).

Wound dressing material	Animal	Size of created wound	Wound closure rate (%)	Total days for full recovery (day)	Wound stimulation mechanism	Biodegradation rate	Refs
Chitosan-based hydrogel with MOF-loaded α -lipoic acid	Male <i>SD</i> rats	$\varnothing = 15$ mm	100	11	Nil	Nil	[15]
Chitosan/poloxamer-based thermosensitive hydrogels containing zinc gluconate/recombinant human epidermal growth factor	<i>SD</i> rats	Nil	100	21	Zn^{2+} and rhEGF	Slow degradation till 20 days	[16]
Sprayable thermosensitive hydrogel coupled with zinc modified metformin	Male C57BL/6 mice	$\varnothing = 8$ mm	100	11	ZnMet could inhibit ROS	Nil	[17]
ZnO-Fe ₂ O ₃ /kaolinite nanoclay/poly-(3-caprolactone)-gelatin	Male BALB/C mice	Area of 2 cm ²	88	14	Nil	Nil	[18]
Bionanofiber chitosan/polyvinyl alcohol incorporating honey and <i>Nepeta dschuparensis</i>	Male Wistar rats	$\varnothing = 2.2$ cm	59.85	21	Nil	Nil	[19]
Thymoquinone loaded chitosan-lecithin micelles	Balb/c mice	1 cm ²	100	16	1, 4 benzoquinone bioactive constituent	Nil	[20]
Carboxymethyl cellulose nanofibril-loaded fucoidan/alginate-based gellan gum	ICR female mice	Full thickness of 8 mm	100	14	ROS	Nil	[21]
Copper ions-substituted hydroxyapatite/polydopamine nanocomposites	Male Balb/c mice	Nil	100	14	Cu^{2+}	Nil	[22]
Polysaccharides from <i>Gracilaria lemaneiformis</i>	Male KM mice	$\varnothing = 0.5$ cm	100	12	Sulfated polysaccharides	Nil	[23]

Table 1.0

An ideal wound dressing material with biodegradable properties and *in vivo* animal toxicological investigation (continued).

Wound dressing material	Animal	Size of created wound	Wound closure rate (%)	Total days for full recovery (day)	Wound stimulation mechanism	Biodegradation rate	Refs
Chitosan-based polyurethane hydrogel	Female Wistar rats	Ø= 6mm	> 90	14	Nil	100% in 8 weeks	[24]
Quaternary ammonium chitosan nanoparticles (TMC NPs)/chitosan (CS) composite sponge	Male Kunming mice	1.5cm×1.5cm	100	16	Nil	58.2% in 5 days	[25]
Ag-PCL/GelMA fibrous membranes	Male <i>SD</i> rats	Ø= 15 mm	> 90	20	Nil	Nil	[26]
Catechol modified quaternized chitosan	BALB/c mice	1.5 cm long	99.4	10	Nil	100% in 6 weeks	[27]
Chitosan biopolymer encapsulated 1wt.% green synthesized binary ZnO/CuO from <i>Calotropis gigantea</i> (<i>C. gigantea</i>)	Male <i>SD</i> rats	Area: 2cm 2cm and 0.2cm depth	86.44	14	Cu ²⁺ and Zn ²⁺	Fully biodegradable in 8 days	"This work"

2. Materials And Methods

2.1. Materials

In this study, zinc nitrate hexahydrate (Acros) and copper (II) nitrate trihydrate (99.5%, Merck), from Darmstadt were used as main precursors. Silver alginate (Ag-Alg) was used as commercial control. Medium molecular Cs flakes (Aldrich) with degree of deacetylation 75–85% and mean molecular weight of 190,000-310,000 Da was used for Cs nanocomposites formulation. The fibroblast cells lines model, L929 used in this work was purchased from American Type Culture Collection (ATCC, USA). Fibroblast was maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Life technologies) and the media was supplemented with other nutrients. The alamarBlue™ cell viability reagent DAL1025 (Invitrogen, United Kingdom) was used for cell viability assay. The MDR pathogen (MRSA 38591) was obtained from American Type Culture Collection (ATCC). Luria-bertani (LB) nutrient medium was purchased from Sigma Aldrich (Darmstadt, Germany) and was used to culture MRSA 38591. Finally, male adult *SD* rats were purchased from Animal Research Centre (ARC), Advanced Medical and Dental Institute, Universiti Sains Malaysia.

2.2. Preparation of lyophilized porous ZnO/CuO-Cs-1wt.% nanocomposites

Green-synthesized dual ionic ZnO/CuO nanocomposites from *C. gigantea* leaf extract and freeze-thawed porous ZnO/CuO-Cs-1wt.% nanocomposites were fabricated according to method described in previous works [28, 29]. *C. gigantea* is also known as giant milkweed or *Lembiaga*.

2.3. Characterization of lyophilized porous ZnO/CuO-Cs-1wt.% nanocomposites

2.3.1. HPLC pre-screening analysis

High-performance liquid chromatograph (HPLC) with PDA Detector (SHIDMAZU HPLC, PROMINENCE LC-20AT) was used to determine the finger prints of phenolic compounds. The *C. gigantea* leaf extract aqueous solution was prepared using deionized water at boiling condition. This pre-screening method was done using mobile phase of acetonitrile solution (A) and 0.1% v/v formic acid solution in water (B) at 30°C with different elution gradients and run times (Method C) as recommended by previous works [30]. The flow rate of the mobile phase was fixed at 0.4 mL/min [31]. Then, 2 µL of diluted extract solution volume was injected. UV peak absorbance was captured at wavelengths of 280 nm and 360 nm. For method C, linear gradient elution was set as: 0–12 min, 85% B; 12–22 min, 75% B; 22–30 min, 85% B.

2.3.2. Physical appearance and pH determination

The physical appearance of fabricated samples was captured through visual inspection. The pH of each type of mixing compound (pure Cs and ZnO/CuO-Cs-1wt.%) was determined using basic bench top pH meter (EUTECH pH 700).

2.3.3. Storage analysis

The stability and bactericidal performance of freeze-thawed pure Cs and ZnO/CuO-Cs-1wt.% (1mm×1mm) was investigated against MRSA at different storage temperature (25 and 4°C) and different storage time (1 and 2 months) [32]. The samples were kept inside air tight glass bottle during the treatment. The bactericidal effect of fabricated samples was measured by reading absorbance of treated sample in 10⁶ cfu/mL bacterial broth culture (200 µL/well) after 24 hours of incubation using microplate spectrophotometer at optical density wavelength of 580 nm. All treatment groups were carried out in triplicate.

2.3.4. Biodegradation study

The degradation (%) of the 6 mm diametrical disc of pure Cs, ZnO/CuO-Cs-1wt.% and positive commercial control (Ag-Alg) samples were monitored at regular time intervals (4, 6 and 8 days) by carefully immersing them in a small air tight glass bottle with 3 mL of phosphate buffer saline (PBS) at physiological pH of 7.4 and incubation temperature at 37°C. The weight loss of the samples over time were evaluated by recording the initial weight before immersion and final weight after immersion. Final weight of the sample at each regular time interval was measured after fully removing the excessive water content from the sample followed by gentle dabbing using tissue paper. This protocol was performed as illustrated by Maiti et al. (2021) [33]. The degradation (%) was determined using the formula:

$$\text{Degradation (\%)} = \frac{\text{Initialweight} - \text{Finalweight}}{\text{Initialweight}} \times 100\%$$

2.3.5. Cytocompatibility assay ISO 10993-5 (2009)

This set of experiment was carried out to evaluate *in vitro* cytocompatibility for ZnO/CuO-Cs nanocomposite using ATCC fibroblast cells lines model, L929. Direct contact method (ISO 10993-5) protocol was applied and cell viability was measured quantitatively via Alamar blue assay. This is a common test method used in evaluating wound healing film [34, 35]. A sample is classified as cytotoxic if the cell viability is lower than 70% [36]. The cell lines were seeded in to 96-well plates at a seeding density of 1 × 10⁴ cells/well [37, 38, 39] and incubated for overnight in the following conditions of 5% CO₂, 37°C and >90% humidity. The test samples with equal size (1mm×1mm) [34, 40] were cut and sterilized under UV for 20–30 mins [41, 42, 43]. The old culture media was removed after formation of confluent and homogeneous cell's monolayers and then, replaced with 200 µL/well of fresh culture medium [39] along with three replicates samples for each treatment according to the direct seeding method [35, 37, 44, 45]. Two biological conditions were carried out. The following controls such as pure Cs control, cell control (L929), commercialized control (Ag-Alg), and a negative control (10% (v/v) DMSO) were presented as well. The cell's morphology and migration were analyzed under inverted microscope after different incubation period of 24, 48 and 72 hours. At last, 100 µL of 10% of Alamar blue solution was placed at respective time intervals and the cell viability absorbance was determined via microplate spectrophotometer at optical density wavelength between 570 nm and 600 nm.

2.3.6. *In vitro* wound scratch assay using L929 cell line

An *in vitro* wound scratch method was employed to study wound closure and cell migration rate of the cell lines according to the established protocol [46, 47]. Briefly, 3×10⁴ cells/well were seeded into 96 well culture plates and incubated for 24 hours in CO₂

incubator at 37°C. A sterile 200 µL pipette tip was used for wound scratch. Suspension containing of pure Cs, ZnO/CuO-Cs-1wt.% and commercial control (Ag-Alg) with size sample of 1mm×1mm in 200 µL culture media were prepared for treatment. Meanwhile, 10% (v/v) of dimethyl sulfoxide (DMSO) was used as negative control. At different time interval (0, 12 and 24 hours), the wound closure distant (µm) and cell migration over time were observed under the inverted microscope and analyzed via ImageJ software. This assay was carried out with two biological replicates with n = 3. Wound closure % and cell migration rate (R_M) were calculated using recommended Equation [1.0] [47] and Equation [2.0] [48, 49]:

Equation [1.0]:

$$\text{Wound closure (\%)} = \frac{\text{Average initial distance scratch} - \text{Average distance scratch after time interval}}{\text{Average initial distance scratch}} \times 100$$

Equation [2.0]:

$$R_M = \frac{\text{Average initial wound width} - \text{Average final wound width}}{\text{Timespan}}$$

2.3.7. *In vivo* wound healing study using *SD* rat model

In this work, total 12 male *SD* rats, aged between 6 and 8 weeks which approximately weighs 200 g to 250 g, were utilized in four different treatment group. The number of the animals per group (n) were based on the method employed by Jamadi et al. (2017) [50], Ismail et al. (2019) [51], Yeng et al. (2019) [52] and Fu et al. (2021) [53]. In the event, animal need to be euthanized due to humane consideration, it shall be replaced to meet the minimum 3 animals per treatment. All experimental animals were approved by USM Institutional Animal Care and Use Committee (USM IACUC) with approval number **USM/IACUC/2022/ (134) (1187)**. Animals were placed in the individual cage (singly house) with corn cob bedding. Standard laboratory diet food 4 mm pellets (Altromin) and unrestricted supply of drinking water were given [54]. Daily water and food intake of all animals were closely monitored. All rats were anesthetized by intraperitoneal injection of 75.0 mg/kg ketamine and 8.0 mg/kg xylazine [54, 55, 56]. Under anaesthesia, the dorsal hair from mid back surface had been shaved carefully using hair clipper and razor.

After that, the skin on the back area were marked on the posterior part of the scapula, and then, using scissors and a surgical blade, the entire skin was removed so that the 2 mm depth of the wound included a derma and a hypoderm, and the wounds were washed with normal saline [55]. The size of the shaved area is more than 4 cm square, and the wound size is 2cm 2cm 0.2cm. Each animal was wounded with one square excision wounds on dorsal individually. Analgesic (Meloxicam) 1.0–4.0 mg/kg subcutaneously administrated for maximum 3 days against *SD* rats for post-surgery. Then the initial wound area which created on Day 0 was photographed and recorded. In next, the sterilized samples were placed over the created wounds and fixed in place using an adhesive biological membrane (Tegaderm™ Transparent Film). The wound that is not treated with bacteria served as Blank or Bare group [57]. The wound that is treated with commercial hydrogel bandage (Ag-Alg) served as positive control group. For other optimized samples pure Cs and biodegradable porous ZnO/CuO-Cs-1wt.% nanocomposites were applied in bare wound of rats.

Wound size created on first day (Day 0) will be set as benchmark. The dressing materials were changed at Days 3, 7, 11 and 14. During the changing of dressings, photographs were taken, and the wound area were measured using a UV sterilized transparent polyethylene sheet. The sheet was kept on top of the wound and area were marked using a marker pen. The marked area was then transferred to graph sheet for getting the exact value through analysis using image J software [54, 55, 58]. After day 14, all animals were euthanized by lab technician with method of asphyxiation using 100% of CO₂ inhalation with flow rate 10–20% of the chamber volume per minute. All major internal organs, including heart, liver, spleen, lung and kidney were harvested after euthanasia for naked-eye morphological observation (i. e., swelling, tumor growth and discoloration) [44, 58]. The cytotoxicity effect of nanosized binary ZnO/CuO in Cs biopolymer against internal organs of *SD* rats can be investigated by comparing treated group with normal bare control group animals [59, 60, 61, 62, 63].

2.3.8. Quantification of multiple metal ion release

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Perkin Elmer OPTIMA 7300DV) was used to quantify the total Zn (Zinc), Cu (Copper) and Ca (Calcium) ions release (mg/L) from ZnO/CuO-Cs-1wt.% nanocomposite. Cs sample labelled

as blank control, while Ag-Alg as commercial control. A standard calibration curve was prepared by running a series of standard solutions ranging from 1 ppm, 10 ppm, 25 ppm and 100 ppm made from stock solution. Square sample (1cm 1cm) of each treatment group was immersed in 20 mL deionized water for 24 and 72 hours at room temperature for metal ion facilitation.

2.3.9. Statistical analysis

Two-way ANOVA test was employed to determine the differences among the different treatment's groups. Data was considered statistically significant if *P* value is less than 0.05. All the test data are expressed as the mean ± standard deviation (SD) of three replicates.

3. Results And Discussion

3.1. HPLC pre-screening analysis

In this pre-screening study, the bioactive constituents such as phenolic and flavonoids in *C. gigantea* leaf extract solution were determined. The graph captured several main peaks corresponding to retention time of 3.937 min, 6.659 min and 6.771 min (Table 2.0). Most prominent and sharp intensity peak of Quercetin bioactive compound was detected at 6.659 min [64] (Figure S1; Supplementary material). Some minor peaks at RT of 3.937 and 6.771 might be attributed to the presence of Vanillin and Benzoic acid, respectively [30]. The FTIR and UV-vis results from previous study agree with the finding of formation of flavonoid compound during green synthesis [28]. In the presence of high content of Quercetin phytocompound in *C. gigantea* leaf extract solution, antibacterial properties and antioxidant activities were further improved [65]. Quercetin bioactive compound belongs to flavonoid group of polyphenols with molecular formula of C₁₅H₁₀O₇. Naturally, it has many promising characteristics such as anticancer, antiviral, antifungal, antimicrobial, anti-inflammatory and antioxidant. In this study, we believed that the Quercetin phytocompound play an important role in promoting synergistic antibacterial activity towards MDR and non-MDR skin pathogens [28, 66].

Table 2.0
Several main peaks corresponding to retention time (RT).

Peak #	RT (min)	Area (%)	Height (%)
1	3.937	10.472	1.916
2	6.659	63.019	89.106
3	6.771	26.269	8.956
4	8.643	0.240	0.022

3.2. Physical appearance and pH determination

Porous Cs and Cs biopolymer containing 1 wt.% of binary green-synthesized nanocomposites (ZnO/CuO) samples, were fabricated by freeze-thawing process appears in white and greyish white, respectively. Both samples show a strong acidic pH between 4.50 and 4.69. Incorporation of 1 wt.% of binary green-synthesized ZnO/CuO nanocomposites in Cs biopolymer slightly increase the pH. Enhanced antibacterial effect and speed wound recovery could be achieved by creating an acidic environment. This could be explained that, an acidic pH level might accelerate wound healing by fighting against wound infection, boosting bactericidal activity, improving re-epithelization and angiogenesis [67]. Previous studies reported that, the transparent acidic Chitosan + 75%Honey sample with pH level of 4.34 had highest ZOI towards *P. aeruginosa*, *S. aureus*, *K. pneumonia* and *S. pyogenes* and it also has highest healing rate of burn wounds [68]. Other than that, ZnO QDs has rapid dissolution and release of zinc ions and excellent bactericidal effect at pH below than 5.5 [69].

3.3. Storage analysis

The storage analysis (Tables 3.0 and Table 4.0) revealed that, the different storage temperature (25 and 4°C) and different storage time (1 and 2 months) of the Cs and ZnO/CuO-Cs-1wt.% samples were able to tune the bactericidal performance against multi-drug resistant pathogen (MRSA). Particularly, the 1 month treated-storage group (ZnO/CuO-Cs-1wt.%) at different storage temperature exhibited a significant antibacterial effect with low optical density between 0.37 ± 0.05 and 0.48 ± 0.06 , while control MRSA strain showed highest absorbance of 0.95 ± 0.04 . The material shelf life might be deteriorated if it is kept at longer period. So far very limited research works only investigated on bactericidal performance of storage condition-dependent wound dressing material [32].

Table 3.0
Absorbance for storage room temperature at different storage time (1 and 2 months).

Stored at room temperature		
Sample	1 month	2 months
Control MRSA	0.95 ± 0.04	0.93 ± 0.01
Cs	0.8 ± 0.05	0.85 ± 0.08
ZnO/CuO-Cs-1wt.%	0.37 ± 0.05	0.49 ± 0.09

Table 4.0
Absorbance for freezing temperature at different storage time (1 and 2 months).

Stored at 4°C inside freezer		
Sample	1 month	2 months
Control MRSA	0.95 ± 0.04	0.93 ± 0.01
Cs	0.55 ± 0.01	0.97 ± 0.02
ZnO/CuO-Cs-1wt.%	0.48 ± 0.06	0.53 ± 0.16

3.4. Biodegradation study

Degradation rate (%) of the prepared biomaterials are depicted in Table 5.0. The degradation rate for Cs, ZnO/CuO-Cs-1wt.% and commercial control samples were 100 ± 0.00 , 100 ± 0.00 and $50.35 \pm 11.20\%$, respectively. The degradation rate of ZnO/CuO-Cs-1wt.% sample was greater than commercial control sample where it could be fully dissolved in 8 days, while commercial control sample degraded partially. As per observed, the percentage of degradation of these samples were time-dependent and increases proportionally. In the present study, we found that the pure Cs sample was degraded partially at 3 hours of immersion time and fully dissolved at 8 hours of immersion time which may indicate that pure Cs without an addition of green-synthesized binary nanocomposites has very weak polymer chain and structural properties resulting in prominent degradation rate. Based on our findings, ZnO/CuO-Cs-1wt.% sample were highly swelled on 1–2 days of immersion time and the water content of the sample cannot be fully dabbed with tissue paper where it might destroy the nanoparticulated polymer structures. However, ZnO/CuO-Cs-1wt.% sample have considerable biodegradation properties which play an important role in eliminating medical waste and create green environment without soil pollution.

Table 5.0
Degradation (%) of the prepared biomaterials.

Sample	Degradation (%)		
	4 Days	6 Days	8 Days
Cs	100 ± 0.00	100 ± 0.00	100 ± 0.00
ZnO/CuO-Cs-1wt.%	17.18 ± 13.44	82.17 ± 5.65	100 ± 0.00
Ag-Alg	1.19 ± 0.32	29.36 ± 20.82	50.35 ± 11.20

3.5. *In vitro* cytocompatibility investigation at different incubation time

The investigation of nanoparticulated polymer biomaterial (ZnO/CuO-Cs-1wt.%) *in vitro* was studied against fibroblast cell line at different incubation time (24, 48 and 72 hours) and the results are depicted in Fig. 1.0. These results demonstrated that, (ZnO/CuO-Cs-1wt.%) sample had no apparent cytotoxic effects (~ 169%) along with normal elongated filopodia cell morphology at 72 hours treatment, indicating that this bactericidal and wound healing agent owned an excellent wound closure rate and cytocompatibility nature. This was owing to the high porosity and improved swelling characteristic of ZnO/CuO-Cs-1wt.% sample which increases absorption capacity of exudate from chronic wounds and provides passages for intake of nutrients and body fluid for wound recovery and re-epithelization [70].

Besides of that, successful control on steady and slow release of free radicals such as $\cdot O_2^-$ and $\cdot OH^-$ from this ZnO/CuO-Cs-1wt.% nanocomposites might present with cytocompatibility properties towards human cells and can accelerate wound healing properties [71]. We believed that, smaller size irregular oval and quasi-spherical shape green-synthesized binary 75ZnO/25CuO-300C nanocomposites might further accelerate controllable amounts of metal ions release and promote cell proliferation [58, 72]. In line with this study, pure Cs sample exhibited cell viability of 129%, which was slightly lower to the (ZnO/CuO-Cs-1wt.%) sample, while the negative control does not promote the cell's augmentation. Collectively, these outcomes exhibited that ZnO/CuO-Cs-1wt.% would be suitable candidates for wound healing application.

3.6. *In vitro* wound healing profile: scratch assay

The wound healing efficacy of an optimized ZnO/CuO-Cs-1wt.% nanocomposites sample was further evaluated against fibroblast cell lines at different time intervals (12 and 24 hours). The findings on wound closure (%) and cell migration rate over time was shown in Table 6.0 and Fig. 2.0. The wound closure for ZnO/CuO-Cs-1wt.% nanocomposite is equivalent to the commercial control (Ag-Alg) which is around 62% at 12 hours. Meanwhile, a slightly improved wound healing extent (45%) were seen in the pure Cs group in comparison with the cell control (L929). This might be linked to the release of positively charged Ca ions (Ca^{2+}) from Cs polymer, which was revealed from EDAX, XRD and ICP analysis [29]. These ions might help in promoting cell proliferation and wound healing [73]. But it was noted that, all treated groups have complete wound closure at 24 hours except negative control has shown no sign of wound healing. Rapid wound closure rate was clearly seen for the ZnO/CuO-Cs-1wt.% treated group. Moreover, ZnO/CuO-Cs-1wt.% treated group has exhibited remarkable cell migration rate of 26.81 $\mu m/h$ at 12 hours. This might be due to the effectiveness of optimized ZnO/CuO-Cs-1wt.% biomaterial in stimulating wound healing and angiogenesis process via synergistic activity with presence of multiple ionic system (Cu^{2+} , Zn^{2+} and Ca^{2+}) [58, 74, 75] and hydrophilic nature of Cs molecules as it was proven before in contact angle analysis [76]. From the above discussion, it was clear that ZnO/CuO-Cs-1wt.% has remarkable wound healing characteristic for biomedical applications.

Table 6.0
Wound closure (%) and cell migration rate for different control and treatment group against fibroblast cell lines. "NA" symbolizes as no wound closure was observed.

Sample	Wound closure (%)	Cell migration rate ($\mu\text{m/h}$)
L929 Control	42.56 ± 7.41	21.8
Cs	45.95 ± 0.76	14.45
ZnO/CuO-Cs-1wt.%	62.35 ± 9.46	26.81
Commercial Control	62.80 ± 5.86	23.39
DMSO Control	NA	NA

3.7. *In vivo* wound healing profile: excisional open wound in animal *SD* rats

In this work, Cs biopolymer containing 1 wt.% of binary green-synthesized nanocomposites (ZnO/CuO) sample has shown remarkable wound closure rate within 14 days. The efficacy of wound dressing material was investigated in the *SD* rats wound model ($n = 3$). Changes on initial wound area at day 3, 7, 11 and 14 were observed and captured as shown in Fig. 3.0. Dry surface and scab formation without accumulation of pus were clearly seen for all created wounds after administration of dressing material. Bare/blank control group and Cs control group exhibited slower wound healing than other treated groups. However, by day 3 the wound healing for ZnO/CuO-Cs-1wt.% treated group showed faster reduction in wound closure ($44.05 \pm 4.77\%$) while bare/blank control, Cs control and commercial positive control treated groups have lower wound closure rate which was approximately between 20.63 ± 7.99 and $28.41 \pm 8.29\%$. This could be explained due to the high porosity and swelling index of porous Cs biopolymer containing 1 wt.% of binary green-synthesized nanocomposites (ZnO/CuO) than Cs control and commercial positive control [29].

The average wound closure rate (%) on day 14 was 75.64 ± 7.83 in the bare/blank control group, 80.86 ± 2.18 in the commercial positive control group, 75.58 ± 2.60 in the Cs control group and 86.44 ± 6.48 in the ZnO/CuO-Cs-1wt.% treated group. Among all groups, wound treated with biodegradable porous ZnO/CuO-Cs-1wt.% nanocomposites provide an ideal wound healing environment and superficial fibroblast proliferation. Thus, low-concentrated Cu, Zn and Ca ions play an important role in stimulating cell migration in open wound [58, 72]. In all treated *SD* rats, there was no evidence of behavioral sign of illness or pain such as guarding, vocalizing, biting, restlessness, abnormal resting position, failure to groom, lack of mobility. All experimental *SD* rats administrated with ZnO/CuO-Cs-1wt.% displayed "0" grimace scale, and there were no treatment related morbidity and mortality. Besides of that, no any abnormalities were seen on the skin, fur, eyes, mucous membrane, stool and urine for all treated group of *SD* rats.

Moreover, the use of ZnO/CuO-Cs-1wt.% is advantageous to increase the material absorption on wound area through melting and slow degradation, whereas the positive control hardly absorbed on wound surface due to the slow degradation as positively captured in biodegradation study (Figure S2; Supplementary material). In addition, all *SD* rats gained their body weight during 14 days of treatment period (Fig. 4.0a). No significant differences were captured in food and water intake for all treated and bare control group during the treatment period (Figs. 4.0b and 4.0c). All harvested major internal organs such as spleen, kidneys, heart, lungs and liver were identically healthy and revealed normal morphological appearance in term of size, shape and colour without any tumor growth and swelling (Fig. 5.0).

Previously, several studies have shown that the formulated wound healing agent such as AMP- and CeON-loaded catechol-modified hydrogel [6], Chitosan-based hydrogel with MOF-loaded α -lipoic acid [15], Chitosan/poloxamer-based thermosensitive hydrogels containing zinc gluconate/recombinant human epidermal growth factor [16] and Ag-PCL/GelMA fibrous membranes

[26] dramatically cured the created initial wound size (0.8–1.5 cm in diameter) within 11 to 21 days. However, this developed biodegradable porous ZnO/CuO-Cs-1wt.% nanocomposites in the current work were found to be best formulation than other reported wound dressing products, since it managed to heal the larger size (square shape: 2cm 2cm) of created wound on day 0 quickly in total 14 days. Conclusively, this developed green wound dressing has emerged a promising potential in biomaterials for open wound healing application.

3.8. Multiple metal ion release

The release of Zn, Cu and natural Ca ion derivatives concentration in deionized water were quantified in line with the material degradation at Day 1 and Day 3. Concentration of metal oxide ions was a very important factor in promoting synergistic killing activity towards skin pathogens. It could be seen from Fig. 6.0 that the dissolution of Zn and Cu ions was slightly affected by the different immersion days of biodegradable porous ZnO/CuO-Cs-1wt.% nanocomposites. The detected Zn, Cu and Ca concentration after immersion of 3 days for ZnO/CuO-Cs-1wt.% nanocomposites sample were 3.23 ± 0.063 mg/L, 0.37 ± 0.014 mg/L and 2.09 ± 0.042 mg/L, respectively. It demonstrates that, Zn and Ca ions release concentration peaked on day 1 and then gradually declined after 3 days. The rate of metal oxide ions (i. e., Zn^{2+} and Ca^{2+}) release slightly reduced with longer immersion days [77]. The detected Zn concentration was significantly higher than Cu and Ca element due to the high reactivity and solubility of ZnO in deionized water. Conclusively, these multiple metal ions derivatives release from nanocomposites could be a viable solution to combat antibiotic resistance by killing the skin pathogens.

4. Conclusion

Development of a synergistic biodegradable ZnO/CuO-Cs-1wt.% nanocomposite has shown an enhanced *in vitro* and *in vivo* wound healing characteristics and migration rate. The wound healing properties greatly improved with multi-ionic systems (i.e., Cu^{2+} , Zn^{2+} and Ca^{2+}). Besides, the degradation rate of ZnO/CuO-Cs-1wt.% sample was greater than commercial control sample where it could be fully dissolved in 8 days, while commercial control sample degraded partially. Thus, technology from this medicinal plant *C. gigantea* especially in development of advanced transdermal ZnO/CuO-Cs wound dressing could provide efficient wound healing response.

Abbreviations

Cs: Chitosan, *C. gigantea*: *Calotropis gigantea*, CuO: copper oxide, ZnO: zinc oxide, ROS: reactive oxygen species, MDR: multi drug resistant, MRSA: Methicillin-resistant *Staphylococcus aureus*

Declarations

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Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to the patent application but are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest.

Authors' contributions

Rabiatul Basria S. M. N. Mydin is the principal investigator who contributed in the concept, idea, experimental design, and writing process and gave final approval of this paper for publication. G Ambarasan Govindasamy carried out all experimental works and prepared the manuscript. Nasrin K Ramtan Gadaime assisted in animal monitoring work. Srimala Sreekantan guides in the material characterization procedures. All authors have given approval to the final version of the manuscript.

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Figures

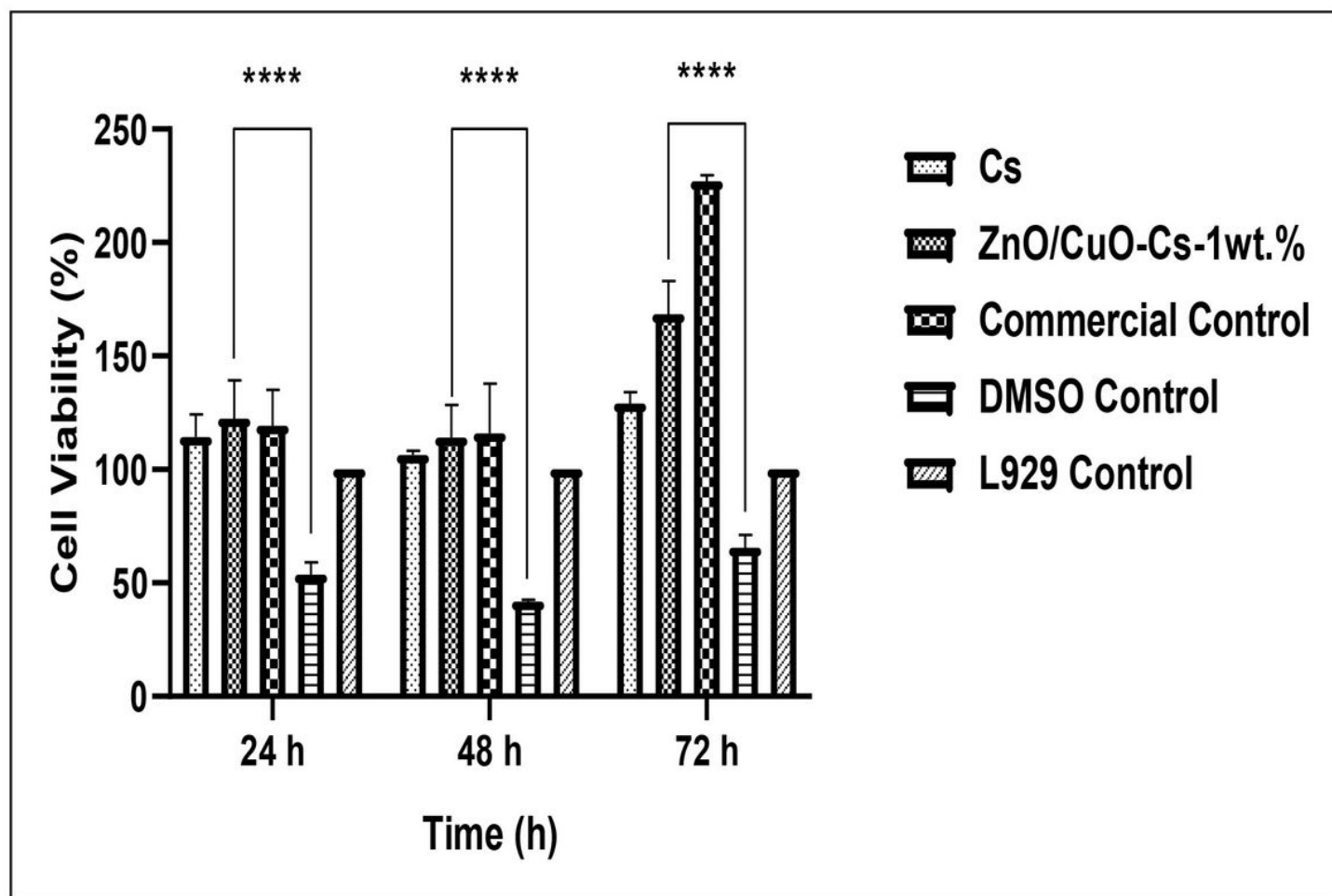


Figure 1

Cytocompatibility investigation on mouse fibroblast cell line at different incubation time.

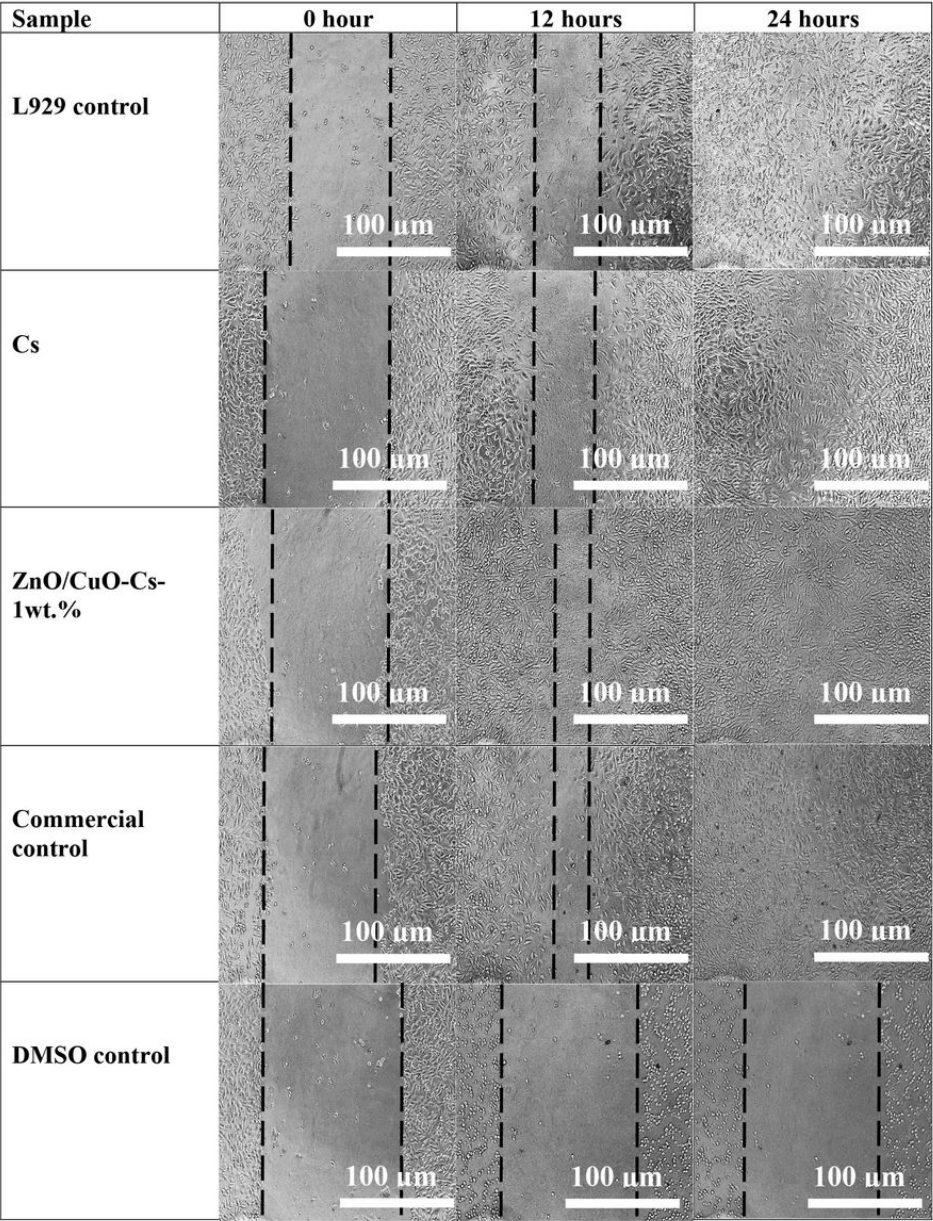


Figure 2

Wound closure and cell migration over time for pure Cs, ZnO/CuO-Cs-1wt.% and commercial control (Ag-Alg).

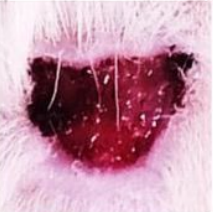
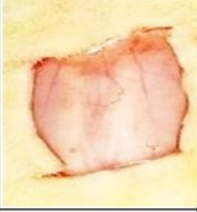
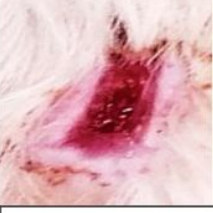
Sample	Day 0	Day 3	Day 7	Day 11	Day 14
Bare/Blank Control					
	0±0.00	20.63±7.99	37.63±1.29	53.25±6.45	75.64±7.83
Commercial Control					
	0±0.00	28.41±8.29	38.35±10.00	59.58±6.95	80.86±2.18
Cs Control					
	0±0.00	21.68±8.82	37.17±5.82	50.24±14.49	75.58±2.60
ZnO/CuO-Cs-1wt.%					
	0±0.00	44.05±4.77	51.9±5.48	73.62±5.69	86.44±6.48

Figure 3

Wound area closure image at day 0, 3, 7, 11 and 14.

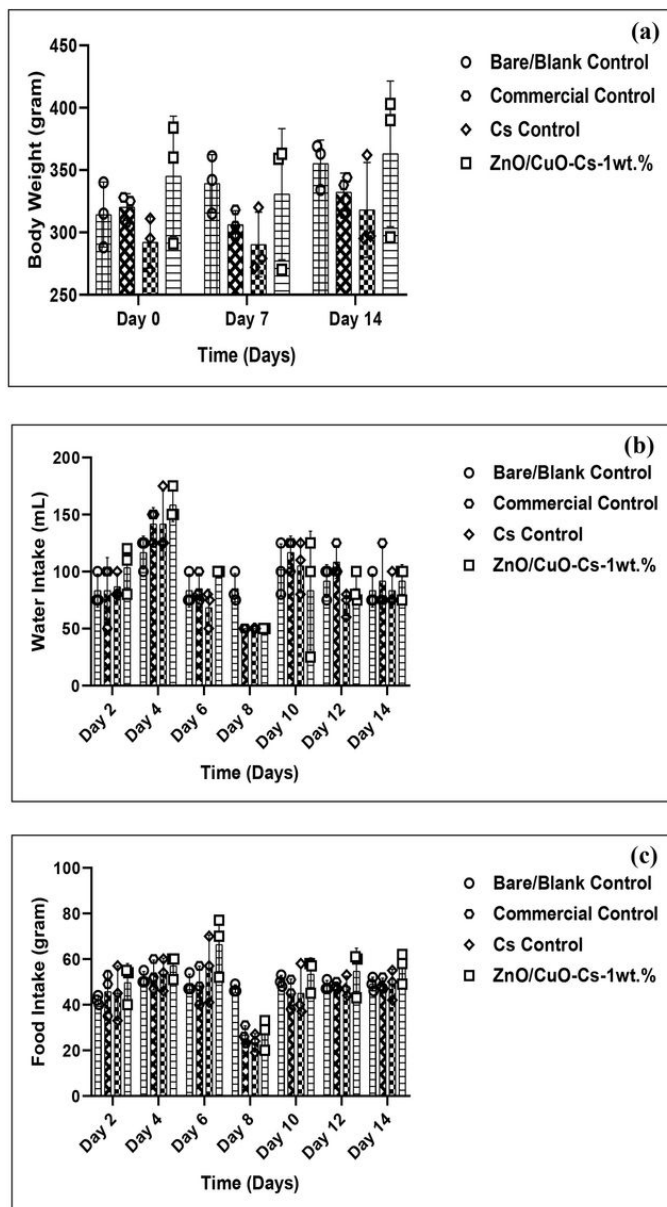


Figure 4

Observed weight gain, water and food intake for all treated and bare control group during the treatment period. (a) body weight gain, (b) water intake and (c) food intake.

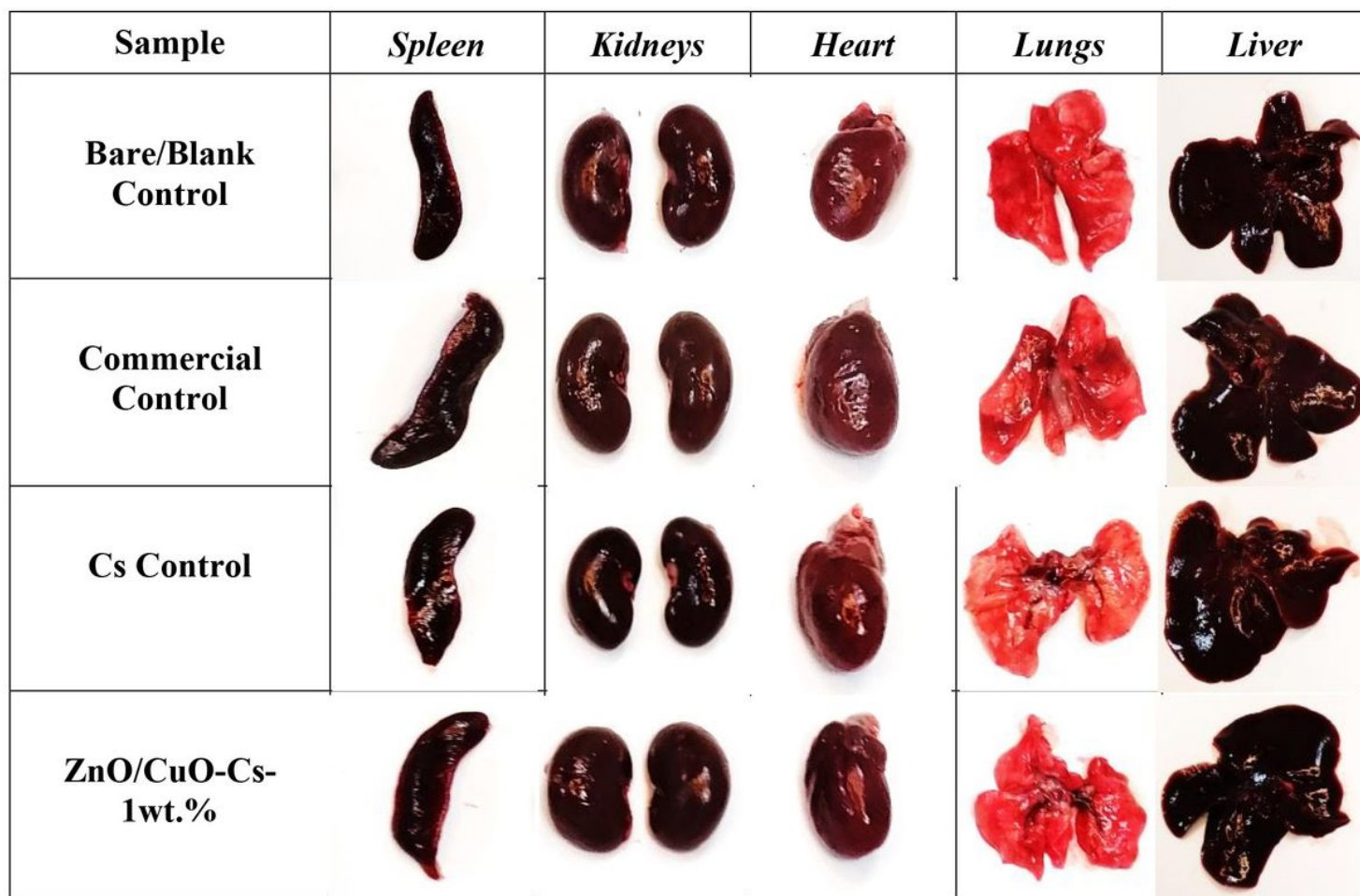


Figure 5

All harvested major internal organs such as spleen, kidneys, heart, lungs and liver after euthanasia.

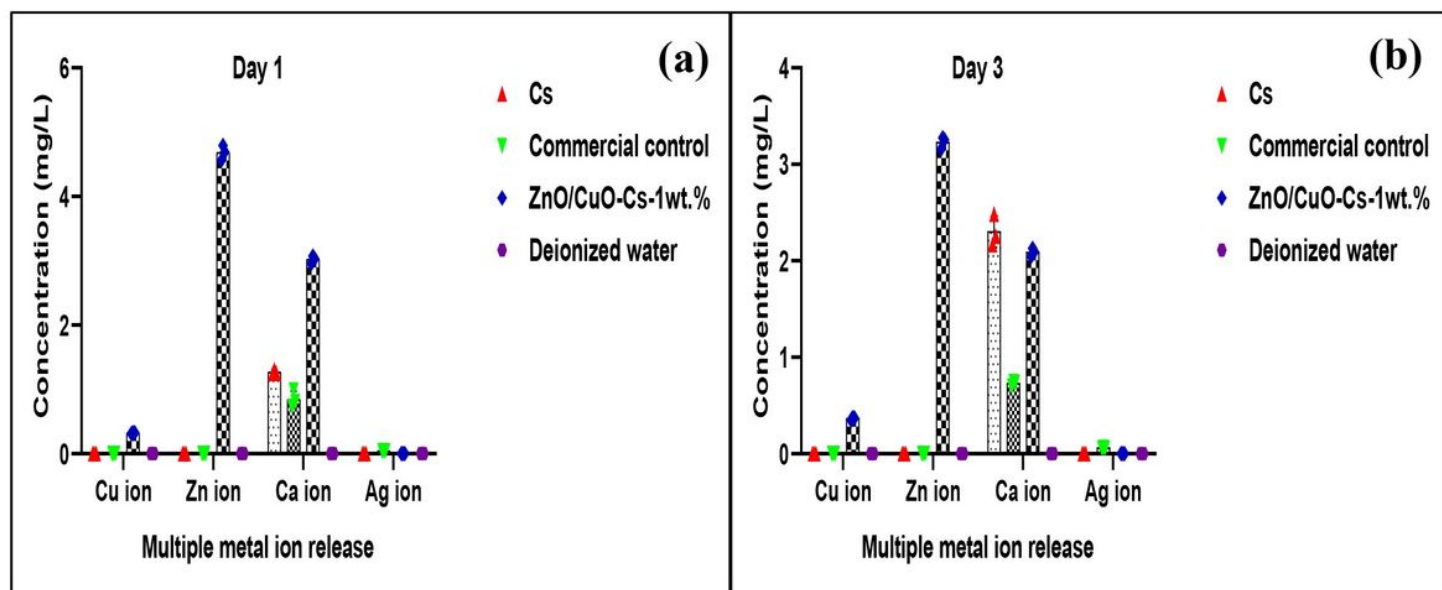


Figure 6

Multiple metal ion release. (a) concentration ions (mg/L) at day 1 and (b) concentration ions (mg/L) at day 3. No metal ion release was detected for deionized water.

Supplementary Files

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