Cuttlebone Fillers for Topical Hemorrhage and Wound Healing: In Vitro Insights and a Suppository Model

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Research

Keywords: Sepia officinalis, Witepsol-based suppositories, clotting agent, skin and tissue regeneration, chitin and chitosan

Posted Date: November 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-102614/v1

Abstract

Background

While the treatment of numerous anorectal disorders remains challenging, this paper aims to add an impact and suggests a product prototype with marine-derived non-toxic cuttlebone fillers for clinical use.

Methods

Elemental composition, hemostatic and antibacterial potential of cuttlebone fillers were tested *in vitro* while comparing with a number of clotting powders. *Witepsol*-based suppositories with lidocaine hydrochloride and cuttlebone fillers were further developed.

Results

Cuttlebone microparticles (CB-1) and modified alkali-treated cuttlebone microparticles (CB-2) were analyzed in terms of hemostatic efficiency and as fillers for rectal suppositories for the first time (Fig. 1). Bioinorganic elements of cuttlebone, such as iron, zinc, copper, calcium and magnesium were found to be supportive for wound healing. Modified, chitosan-enriched cuttlebone filler showed decrease in clotting time by 20%. Cuttlebone fillers demonstrated no antimicrobial activity against *S. aureus* and *P. aeruginosa*. Suppositories with cuttlebone fillers have demonstrated favorable characteristics, such as melting point in a 36.0–37.0 °C temperature range, dissolution time no longer than 30 min and gradual release of the anesthetic drug.

Conclusion

Based on the primal, though essential to conduct *in vitro* test results, application of cuttlebone fillers could open a new page in the development of successful naturally-based hemostatic and wound healing products.

Background

According to traditional practices of Chinese and Indian medicines, cuttlebone has been used to treat various ailments [1, 2]. Regrettably, nowadays previously attained knowledge is left out of consideration. Contemporary research is mostly focused on the use of cuttlebone for bone tissue engineering [3-9], while its wound healing potential is rarely mentioned. Jang and co-authors [10] were the pioneer researchers who described the effect of cuttlebone chitin-based extract for wound healing. In their study, increased re-epithelization of rats' burn injured skin and reduced expression of pro-inflammatory cytokines were observed.

Cuttlebone is an internal skeleton of marine mollusk *Sepia* officinalis, class *Cephalopoda*[11]. Main constituents of cuttlebone are aragonite – a polymorph of calcium carbonate, polysaccharide *β*-chitin and trace minerals [12]. In average, the amount of chitin in cuttlebone is too low to use it as an effective

source material for chitosan production as compared with other crustaceans and cephalopods like shrimp, crab, crill, lobster or squid. Those are rich in chitin and therefore are cost-effective to use for the preparation of commercially employable chitosan-based materials for biomedical and pharmaceutical applications, food industry or wastewater treatment.

Within the last decade, international patents submitted by Chinese researchers disclosed a number of recipes from Chinese medical heritage where cuttlebone is an ingredient in the composition of healing mixtures, ointments and pills. Cuttlebone has been mentioned in Chinese traditional medicine for bleeding control [13]. Empirical practice of nowadays sailors has shown promising results of cuttlebone for epidermal wound healing and bleeding control. Usually, on a boat, fresh cuttlebones were dried in direct sunlight for several days, then crushed and powdered, and later applied to skin wounds when necessary. Continuing the empirical practice, cuttlebone was used for wound healing by sailors and their families for decades[1].

Despite the fact that *powder* is considered as one of the simplest pharmaceutical dosage form to prepare, it could serve as a basis for further developments. For instance, powdered materials are used for making solid dosage forms like effervescent tablets, tablets, granules, capsules or dry-powder inhaler; liquid dosage forms like suspensions; and semi-solid dosage forms like suppositories, suspended gels, creams or liniments.

The retrospective analysis of Medicare beneficiaries (2018) identified that more than eight million people had wounds with or without infections [14]. Bleeding is the most common symptom in grades 1 and 2 hemorrhoids, while infection in a perianal area may present as different types of abscesses, perineal sepsis, fistulae which commonly occur in patients with acute leukemia [15, 16]. Suppositories containing local anesthetics agents, steroids, astringents, vasoconstrictors, protectants, antiseptics, keratolytics, veno-tonics, coagulants of necrotic tissues, analgesics, herbal (rhubarb, aloe) [17] and animal (shark liver oil) components are known to be used for the treatment of anal disorders. While the treatment of numerous anorectal disorders remains challenging, this paper aims to add an impact and suggests a product prototype for clinical care.

This study aims to investigate cuttlebone materials suitable for topical application in wound healing and bleeding control, as well as to develop *Witepsol*-based suppositories for anorectal disorders with cuttlebone fillers.

Methods

Aim

This study aims to investigate cuttlebone materials suitable for topical application in wound healing and bleeding control, as well as to develop Witepsol-based suppositories for anorectal disorders with cuttlebone fillers.

Design

To deepen the topic, following objectives were achieved: i) modification of the original cuttlebone material by means of alkaline deacetylation; ii) characterization of both original and modified materials by means of elemental composition and antibacterial potency, iii) testing of cuttlebone materials *in vitro* in comparison to other powders/granules as for a hemostatic effect; and iv) subsequent development and testing of the suppositories with two cuttlebone fillers.

Commercial hemostatic chitosan-based product *Celox*[1]; bentonite as a prototype of a clay-based hemostatic agent; talc as a negative control[18]; and calcium carbonate (as cuttlebone contains around 90% of aragonite[11]) were used in hemostatic tests for comparison.

Setting of the study

Materials

Cuttlebone was purchased from Vital Pet Products Ltd (United Kingdom). Citrated samples of leukocyte-thrombocyte mixture from concentrated blood (further identified as citrated blood) were donated by National Blood Centre, Vilnius, Lithuania according to the institutional agreement No: 8-90 (23rd of April, 2018) between the National Blood Centre (Vilnius, Lithuania) and Kaunas University of Technology (Kaunas, Lithuania). *Celox* (Medtrade, United Kingdom) samples were kindly donated by Lithuanian Armed Forces Dr. Jonas Basanavičius Military Medical Service (Kaunas, Lithuania) and Lithuanian Special Operations Force (Vilnius, Lithuania). Talc, *Witepso*l H35, solid paraffin, *Carbopol* and lidocaine hydrochloride monohydrate (Farmalabor, Italy), calcium carbonate (Sigma-Aldrich, Germany), lactic acid (Merck, Germany), bentonite (Eurochemicals, Europea) and McFarland 0.5 barium sulphate standard (Liofilhem, Italy) were used.

Electronic laboratory notebook platform was not used. Summary graphic illustration was created in BioRender.com.

Preparation of cuttlebone materials

Whole cuttlebones, including dorsal and lamellar parts, were crushed and milled in an agar mortar; the fraction (particle size 32–45 μm) was collected using sieves. Two types of cuttlebone powders were used in this study: 1) untreated cuttlebone microparticles (CB-1), and 2) cuttlebone microparticles treated with 40% NaOH solution at 80.0 °C for 6 h under continuous stirring (CB-2). After alkaline treatment, the material was thoroughly washed with cooled decarbonized distilled water until neutral pH and dried in a thermostat (Binder ED series 53, Binder GmbH, USA).

X-ray fluorescence analysis

The elemental composition of powdered samples was evaluated by X-ray fluorescence (XRF) spectrometric analysis (Bruker X-ray S8 Tiger WD, Bruker AXS GmbH, Karlsruhe, Germany) and employing

Spectraplus quant-express method.

Microbiological assay

The concentrations of bacteria cultures were adjusted by comparing them to the concentrations in standard tubes with McFarland turbidity of 0.5 (1.5×108 CFU). Muller-Hinton agar powder was used as a culture medium. The inhibiting activity was tested by the disc diffusion method and in two different ways. First method for testing antibacterial activity was adapted from Immanuel et al. [19] where antimicrobial activity of a marine shell powder was tested and second method where the suspension from demineralized cuttlebone powder was tested. For the first method, suspensions of cuttlebone powders were prepared in 2%, 4% and 6% (w/v) concentrations by using sterilized distilled water. Sterile paper discs (6 mm diameter) were impregnated with the respective suspensions for 5 days in sterile glass jars that were kept at 25±0.1°C temperature in an incubator shaker (KS 4000 i control, Werke GmbH & Co. KG, Germany) at 80 rpm. Further, the discs were placed in an agar medium by using a sterilized forceps. For the second method, suspensions from demineralized cuttlebone powder were prepared by adding 5% lactic acid. The amount and concentration of lactic acid for demineralization of $CaCO_3$ in cuttlebone material was calculated according to the Equation 1 and maintained at pH=6.5:

$$CaCO_3 + 2CH_3CH(OH)COOH \rightarrow (CH_3CH(OH)COO)_2Ca + CO_2\uparrow + H_2O \quad (1)$$

The obtained opaque solution was stirred at 50 rpm while using a magnetic stirrer for 2 h. Wells in an agar medium were filled with the solutions by using a micropipette. For both methods, the zone of inhibition was observed after the incubation of Petri dishes at 37.0 °C temperature for 24 h. Four samples were tested simultaneously for each sample concentration and bacterial strain.

Exothermic reaction test in vitro

Exothermic reaction was tested *in vitro* according to Li et al. [20]. Five (5) g of sample powder were placed into a plastic tube and 5 mL of distilled water were added. The temperature reading was recorded before each testing and after 1 min when water was added to the powdered material. Mercury thermometer (Labothermometer, Amarell GmbH&Co., Kreuzwertheim, Germany) with a measuring range from 0.0 to +30.0 ±0.1 °C was inserted into a 25 mL plastic tube. All tests were made in triplicate and the average meaning ± standard errors of deviation (SED) were recorded as results.

Water contact angle

Powdered samples (0.3 g) were pressed into a pellet using a tablet press machine. Contact angles were measured using Theta Light optical tensiometer (Biolin Scientific, Sweden). Data for each sample type (n=5) was recorded within 10 sec and the mean values of left and right contact angles were obtained. The results are expressed as an average of mean contact angle of 5 measurements estimated within 10 sec ± SED.

Coagulation assay

Coagulation assay was performed by testing blood clotting capacity and blood coagulation rate. Blood clotting capacity was tested as described by Liu et al. [21] with some modifications. Briefly, 200 µL of citrated blood were added to 10 ±0.2 mg of each powdered sample in a 6-well plate. To initiate blood clotting, 100 µL of 0.025M $CaCl_2$ solutions were then added and samples were incubated in a thermostat at 37.0 °C for 10 min. After incubation, 15 mL of distilled water were carefully added to each sample without disrupting a formed clot. Ten (10) mL of the resulting hemoglobin solutions from each well were taken and centrifuged at 1000 rpm for 1 min, followed by incubation of the supernatant at 37.0 °C for 1 h. The absorbance of the resultant solutions was measured at 540 nm by using a spectrophotometer (Varian Cary 50 UV-VIS, Varian Inc., Netherlands). The absorbance of blood (200 µL) in 15 mL of distilled water (without any powders added) was used as a reference value. For the quantitative evaluation of clotting ability, the absorbance of hemoglobin from an aqueous solution was taken into consideration, while hemoglobin entrapped in the samples was not counted. All measurements were carried out in triplicate and the average meaning ± standard errors of deviation (SED) were recorded as a result.

Blood coagulation rate was measured imitating the Westergren method and a dynamic rheological experiment as described by Periayah [22]. Blood coagulation rate was measured by observation of clot formation (aggregation of red blood cells, RBCs) during inversion of Eppendorf tubes within time points. For testing of blood coagulation rate, 0.003 ±0.0004 g of a measured powdered sample was poured directly into 1.5 mL Eppendorf tube. Citrated blood was incubated at 37.0 °C for 30 min. After 30 min, 200 µL of blood sample was added to each Eppendorf tube with a powdered sample and coagulation was initiated by adding 100 µL 0.025M $CaCl_2$ solution. Blood coagulation time was recorded every 30 sec, and after 10 min the test was stopped. Citrated blood mixed with a $CaCl_2$ solution was used as a control. Each type of sample was tested in triplicate simultaneously. The results are expressed as an average mean of 3 measurements ± SED.

Visualization

Clots obtained after clotting test were washed with distilled water, frozen and lyophilized (Christ Alpha 2-4 LSC, Osterode am Harz, Germany). Scanning Electron Microscopy (SEM) (FEI Quanta 200 FEG, Oregon, USA) was used to obtain microphotographs of the clot surface. Summary graphic illustration (Fig. 1) is created with BioRender.com

Development of Suppositorieswith cuttlebone fillers

Suppositories were prepared by the pour molding method. Lipophilic base *Witepsol* H35 (W-H35) and solid paraffin (72% and 14% in a total formulation, respectively) were melted at about 70 °C temperature in a hot water bath. Further, Carbopol (2%), lidocaine hydrochloride (2%) and cuttlebone material (CB-1 or CB-2, respectively) were incorporated and mixed using Unguator 2100 (GakoInternational GmbH, Germany) at 5,000 rpm for 5 min; poured into a disposable polyethylene molds (Eprus Czesław Gornowicz S.K.A., Poland), and cooled at room temperature (no hasty freezing). Weight uniformity (n=20) was considered proper when falling within ± 5% of the average value.

Characterization of suppositories with cuttlebone fillers

The open capillary tube method was used to find the melting point according to the description in the European Pharmacopeia. Suppository sample was placed in a cylinder and the temperature of water in the environmental medium was raised by 1°C temperature per min. A temperature point of the first drop of the melted base was recorded. For density determination, a glass cylinder was filled with 10 mL of distilled water. A suppository sample (2.0 g) was immersed into water, and the reading of the increased volume is recorded and the density was calculated.

For the determination of dissolution time, a cylindrical glass with distilled water was put into a water bath (37.0±0.1 °C), and temperature inside a glass is equilibrated. A suppository sample was immersed into a cylindrical glass and trapped slightly to avoid flotation. The content of a cylindrical glass was stirred at 120 rpm until the dissolution of the sample. A time span no longer than 30 min was considered as optimal.

Release of lidocaine hydrochloride was performed by using the basket method (Erweka DZT, Erweka GmbH, Germany) according to the European Pharmacopeia. The basket was rotated at 120 rpm in 500 mL phosphate buffer saline (PBS) (pH=6.8, 37.0±0.5 °C). At predetermined time points, aliquots of 5 mL were withdrawn and filtered, while the volume of the dissolution fluid in a dissolution apparatus flask was compensated immediately by adding the same volume of PBS. The recording of drug concentration in aliquot was measured spectrophotometrically at 265 nm and calculated by using the standard curve (0.5, 0.25, 0.0125 and 0.0625 mg/mL; R^2=0.9925). Data was expressed as the mean value of 3 tests ± SED.

Results

Characteristics of cuttlebone materials

Two types of cuttlebone materials were prepared and analyzed: CB-1 and CB-2 powders. In this work, CB-1 is assumed as a “minerals/chitin” complex, and thus reveals original characteristics of the material. CB-2 powder is a new modification and could be assumed as a “minerals/chitosan” complex (Fig. 2).

According to the results of the elemental analysis (Table 1), zinc, magnesium, potassium, iron and copper were found in both cuttlebone materials. In contrast to bentonite, no aluminum was found in cuttlebone samples[1]. Oppositely to the expectations, all the samples showed no antibacterial activity against the selected bacteria strains. Zones of bacterial growth inhibition for cuttlebone materials were not observed for *S. aureus* and *P. aeruginosa*.

Cuttlebone materials in terms of hemostatic potency

All tested materials showed temperature increase while conducting the exothermic reaction test, however values varied depending on a composition. Minimum temperature increase was observed for CB-1 (0.1±0.1°C), and noticeably higher temperature increase, but within warrantable limits, was observed for

CB-2 (1.1±0.2°C), *Celox* (1.3±0.3°C) and bentonite (1.3±0.2°C). Temperature increase for calcium carbonate and talc were 0.2±0.1°C and 0.3±0.2°C, respectively.

Wetting profile of CB-2 turned towards more hydrophilic if compared with CB-1 (55.7±2.8° and 90.4±1.9°, respectively) probably due to chitin deacetylation and deproteinization. *Celox* appeared to be most hydrophobic material (105.1±2.9°) mainly because chitosan–water interaction induces swelling which impedes water penetration into material layers. Talc and bentonite were the most hydrophilic material (14.8±0.8° and 15.6 ±2.5°, respectively) among all tested samples. Contact angle (°) for calcium carbonate was at 74.4 ±1.3 point.

All values of hemoglobin absorption were significantly low if compared with the control sample (Fig. 3). Herein, lower absorbance value indicates a higher capacity of a sample to entrap hemoglobin and more likely to initiate clot formation from the perspective of protein (hemoglobin) adsorption [21, 23]. As compared with the control (8.8±0.2 min), CB-1 showed no noticeable effect on blood coagulation rate (8.7±0.2 min). Clotting time of CB-2 decreased by 1.8 min and showed a better result in comparison with *Celox* (8.0±0.35 min). Clotting time of bentonite was more than two-fold shorter (4.2±0.2 min) and showed correlation with the results obtained by Baker et al. [24]. Clotting times of talc and calcium carbonate were 6.2±0.2 min and 8.3±0.2 min, respectively.

An additional word file shows the results of hemostatic tests in more detail [see Additional file 1].

SEM microphotographs of clots with cuttlebone

According to macroscopic observation all samples formed clots within 20–30 min of incubation. Fibrin matrix formation, recognizable as a threadlike mesh, was clearly visible on the surface of both cuttlebone materials and *Celox* (Fig. 4). Mostly oval-shaped erythrocytes were visible on the surface of cuttlebone samples, indicating non-disturbance of RBCs morphology during contact with cuttlebone microparticles (Fig. 5). Structured aggregates of RBC on CB-1, CB-2 and *Celox* were visible (Fig. 5a–c). Formation of erythrocyte aggregates was most likely induced by chitosan fragments and, as a result, additional fibrin links were formed. Single erythrocytes were visible on a surface of bentonite, talc and calcium carbonate (Fig. 5d–f). Distorted plate aggregates were visible for talc and calcium carbonate (Fig. 5e, f).

Suppositories with cuttlebone fillers

Suppositories with cuttlebone powdered materials CB-1 and CB-2 were prepared by pour molding technology and choosing *Witepsol H35* (W-H35) as a base: W-H35/CB-1 and W-H35/CB-2. Both formulations showed melting points agreeable to the *European Pharmacopeia* recommendations: 36.2±0.1 °C and 36.3±0.1 °C, respectively. The average weight of prepared suppositories was found not to exceed the established limits for suppositories as single-dose preparations: 1.85±0.1 g and 1.86±0.1 g, respectively. Density (ρ) was measured as 0.16±0.01 g/cm^3 and 0.11±0.04 g/cm^3, respectively. According to the European Pharmacopeia, dissolution time for suppositories in the physiological environment (37.0

°C) needs to be less than 30 min. Both suppository formulations satisfied the requirement: 26±2 min and 27±1 min, respectively.

Gradual release of lidocaine hydrochloride into dissolution medium was observed within 40 min while showing *in vitro* early onset of drug activity and the appropriate drug-release profile (Fig. 6). Drug release profiles of prepared cuttlebone-filled suppositories were found to be similar to the drug release of a commercial suppository (*Doloproct*). Lidocaine hydrochloride release into dissolution medium was the lowest for W-H35/CB-2.

Discussion

Modification of the original cuttlebone material *via* alkaline deacetylation allows to convert inward chitin into chitosan – a well-known clotting agent and wound healer of a marine origin. Alkaline deacetylation is a conventual procedure for chitosan preparation[25] form crustacean waste, while skipping the demineralization step in the same procedure for cuttlebone allow to retain its bioinorganic elements and aragonite. This gives a double advantage because bioinorganic elements of cuttlebone are beneficial for wound healing while a dominant amount of aragonite creates a mechanical barrier for bleeding site (will be discussed in details later).

Chitin and its derivative chitosan are both linear co-polymers consisting of 2-acetamide-2-deoxy-*β*-D-glucopyranose and 2-amino-2-deoxy-*β*-D-glucopyranose, which are connected by *β*(1→4) linkage. The characteristics of chitin, as in CB-1, for biomedical applications have been thoroughly studied. Chitin is a non-toxic and biodegradable natural polymer, used as a matrix in tissue engineering enhancing the surrounding tissue ingrowth and avoiding scar formation. Chitin membranes have shown antimicrobial and fibroblast growth activity. Chitin monomer *N*-acetylglucosamine controls collagen synthesis and improves granulation thus facilitates wound healing [26, 27]. Chitosan (as in CB-2) is known for antimicrobial activity, good biocompatibility, biodegradability, non-toxicity and solubility in acidic aqueous solutions.

So far, scant investigation on the cuttlebone antimicrobial activity has been performed. However, despite the favorable composition which includes Zn, Cu, chitin and/or chitosan and more so, despite the results of other studies showing antimicrobial activity of cuttlebone – in our study the fact was not proven. Antimicrobial activity of CB-1 and CB-2 powders was tested in two ways by using: 1) paper disc diffusion method and 2) fully demineralized cuttlebone materials while imitating normal skin pH in a 4.5–6.5 range. In order to emphasize the pH influence, the second method was performed at pH=6.5 because lower pH could affect the results to positive values due to pH-sensitivity of bacteria itself, but not because of the antimicrobial potency of the samples. Strains of bacteria found on the skin surface, such as *Staphylococcus aureus* and *Pseudomona aeruginosa*, were chosen for the study.

A few theoretical models have been proposed for the explanation of the antimicrobial activity of chitosan. One of them is explained by the electrostatic interaction occurring between the negatively charged zones

of the bacterial membrane and the protonated NH^{3+} in the acidic medium; NH^{3+} groups block the connections of the membrane with Ca^{2+} and thus determine the death of pathogenic cells. Free amino groups that are present in the chitosan structure determine the antimicrobial activity in aqueous solutions below their pKa value (6.0<pH>6.50). Another proposed mechanism explains that chitosan binds with microbial DNA, and the chelation of metals, suppressing the growth of a bacterial cell, occurs[28, 29].

The antibacterial action of zinc and copper against *Staphylococcus aureus*, *Escherichia coli* and *Bacillus* has been already proven [30]. Even though the presence of Zn and Cu and the antimicrobial potency of charged chitosan in cuttlebone composition at pH=6.5 was anticipated, the evaluation of antimicrobial activity showed neither bactericidal nor bacteriostatic activity. Concentration of Zn in the cuttlebone composition (Table 1) is negligible, thus it probably could not manifest positively for antimicrobial activity as in the study by Beherei *et al* [31]. Interestingly if for bone tissue studies, Dogan and Okumus [3] revealed that cuttlebone "is associated with decreased formation of free radicals in soft tissue, and it allows bone healing without causing oxidative stress." In their study, according to the data on biochemical and histological parameters, no inflammatory or foreign body cells were observed and did not cause (re)infection after implantation of non-modified cuttlebone block into a bone defect surrounded by soft tissue.

Other studies focus on the polysaccharides extraction from cuttlebone using methanol and ethylenediaminetetraacetic acid – both potential antimicrobic agents [32-34]. Therefore, successful results on antimicrobial activity of polysaccharide extracts from cuttlebone have been achieved against gram-positive and gram-negative pathogenic bacteria and fungi.

Elemental composition of commercially available cuttlebone, CB-1 in Table 1, is similar to the results for cuttlebone from different coastal zones where biomedical safety aspects were also discussed [4]. Components of cuttlebone have a favorable impact on wound healing. For instance, zinc is an important trace element for a number of living organisms, because it is a structural component of proteins which positively affects their functions. It appears to be one of abundant transition metals in blood composition, binding plasma proteins and modulating their structure and functions by responding to a dynamic microenvironment. By this, zinc could be considered a relevant mediator of hemostasis and thrombosis [35]. Studies have shown that zinc cations are an important cofactor in Factor XII contact activation. Therefore, zinc particles may have an impact on a coagulation process. Magnesium suppresses skin inflammation, while a duet of magnesium and calcium influences cell proliferation and differentiation. Iron deficiency is associated with deceleration in wound healing. Copper modulates melanin synthesis and stimulates maturation of skin collagen. Trace amount of copper in cuttlebone composition is related to the protein hemocyanin which is essential for respiration of cuttlefish [36–30].

Elemental composition of clays, like bentonite, commonly includes aluminum. Aluminum was found in talc, zeolite [21] and *QuikClot*[21] (Table 1). Aluminum is known as a suppressant of collagen synthesis, especially if calcium and magnesium are deficient [37]. This fact could be the main cause for negative

impact of aluminum-containing materials when they come into contact with injured skin, including heat generation and problematic wound healing.

Medicine constantly refers to *pros et contras* for the use of naturally-based materials in bleeding control. Most dramatic adverse effects of naturally-derived hemostatic agents are in-contact exothermic reaction, insufficient biocompatibility and difficulties of agent removal after a surgery. Heat generation, as a result of an exothermic reaction, is the most frequently encountered adverse effect of aluminosilicates (*QuikClot* and *Combat Gause*). Fibrin-based hemostatic agents (fibrin sealants, *Tisseel*; platelet gels, *Vitagel*) are known for the transmission of infectious diseases that are common in biological materials. Clay-based products (bentonite, kaolin and smectite products, such as *WoundStat*[24, 38]) may be a cause of distal thrombosis due to occlusion of arterial flow. Difficulties in removal of post-reacted portions of a material have sometimes been resulted when using mineral-based products [39].

“Test-tube” experiments prior to cell-culture and *in vivo* tests are important when new prototypes are first introduced. As was stressed by Wiegand et al. [40], “the application of controlled *in vitro* techniques might serve as a screening tool in the development of new hemostatic agents.” Nowadays, worldwide research is concentrated on comparability between results of *in vitro* and *in vivo* tests [41, 42]. In contrast to conventional belief that *in vitro* tests should be a “mirror reflection” to animal models, true value of forehead *in vitro* testing is to complement a whole picture and benefit from the interpretation of the results in a multidisciplinary format.

So-called “exothermic reaction” is an undesirable result from a contact between a hemostatic agent and a bleeding site. However, it is a quite often, complex physical-chemical-biological reaction of a living organism. It can cause pain, discomfort, tissue burns, necrosis, and therefore aggravates or severely disrupts proper wound healing. Elemental composition of a hemostatic agent determines a degree of an exothermic reaction, because specific elemental components, mainly aluminum, could cause excessive and accelerated absorption of water at a bleeding site. Subsequently, heat is generated as a result of anomalous accelerated formation of a clot [43]. The reaction originates from rapid dehydration of aqueous blood components followed by an increased local concentration of clotting factors. As a result, skin burns or even tissue necrosis could occur.

Therefore, hemostatic materials showing in-contact low temperature increase are highly desirable. Necrosis, as a worst scenario, was observed during the *in vivo* study by Li et al. [44]. In their study, the temperature increase of zeolite granules and *QuikClot* was 6.9±0.4°C and 44.6±1.0°C, respectively. The authors explained the mechanism of heat generation by excess of Ca^{2+} ions (11.4%) in a composition of *QuikClot*. In comparison to our study, the amount of calcium in cuttlebone powders was five-fold higher: 50.7% and 50.9% for CB-1 and CB-2 powders, respectively (Table 1). However, temperature increase for cuttlebone materials was negligible: 0.1±0.1°C and 1.1±0.2°C, respectively. Calcium in cuttlebone composition is in an aragonite form (i.e. calcium carbonate – a calcium salt with low solubility in water), thus only a negligible portion of ionized calcium in aqueous medium could appear in a short period of time, time period which is necessary for clot formation. Moreover, following the principle of a coagulation

cascade, the threshold level of Ca^{2+} is essential for clotting to start [24]. By taking into consideration all the above-mentioned facts, excess heat generation of *QuikClot*[44] should be explained by other reasons, most likely due to aluminum in its composition.

In terms of hemostatic properties, CB-2 clotting time was shorter by 20.5% if compared with a control sample. The value correlates well with data for chitosan and some commercial products, such as *Gelfoam*, *Surgicel* and dehydrated *QuikClo*t [44].

Liu et al. [21] analyzed blood clotting ability of halloysite nanotubes. In their study, the absorption value of halloysite, a natural inorganic material, was similar to a control sample, demonstrating poor ability to affect blood clotting. However, it needs to be made apparent that blood clotting ability test based on hemoglobin absorbance measurement is more relevant for sponges, fiber mats and other 3D porous materials, because it shows capacity of a sample to absorb blood by volume. Generally, higher absorptive values are found for porous materials as compared with powdered materials [45-47] and that was the case in this study too.

One should appreciate the dual nature of blood: hydrophilic and hydrophobic characteristics in regards to water content and formed elements of blood, respectively. Hydrophilicity of a material is usually characterized by measuring its water contact angle. A hydrophilic surface of a material promotes coagulation cascade, because human blood as a hydrophilic substance (contains approximately 83% of water) and could permeate into a hydrophilic material much easier and faster [48]. Thus, in terms of surface wettability, clotting agents with lower contact angle (hydrophilic) are advantageous. However, only moderate surface hydrophilicity could be deemed as appropriate. For example, in this study contact angles of calcium carbonate and talc are too low (15.6±2.5° and 14.8±0.8°, respectively) to accept them as suitable ones for hemostatic applications.

On the other hand, vast research has been dedicated for studying blood coagulation at biomaterial interface, where blood coagulation and platelet adhesion are examined as a main downside to using implantable medical devices. Herein, hydrophobicity is a determining factor of protein adsorption due to surface chemistry and charge – more proteins will adsorb to hydrophobic surfaces. Blood proteins, such as albumin, fibrinogen and Factor XII, are more adherent onto hydrophobic surfaces and therefore mediate platelet adhesion and thrombus formation. Factors such as contact time, topography and roughness, surface free energy and/or functional groups are the characteristics of a material which could also affect an eventual result [23, 49].

Chemical and physical characteristics of surfaces induce the dynamics of blood protein adsorption onto an artificial surface. Hence, plasma proteins by themselves initiate subsequent clot formation by modulating a number of reactions [23]. Powdered material could enhance clot formation by absorption of blood fluids, acts cohesively and adhesively, thus accelerating agglomeration of cells and stopping hemorrhage by clot formation [18, 50]. Similarly, cuttlebone while crushed into a powder, has an ability to form a mechanical barrier.

In summary, presence of aluminum in some clay-based or aluminosilicate hemostatic agents, in parallel with their deficiency in calcium and magnesium, obviously argues against proper wound healing. Both cuttlebone materials are rich in various bioinorganic elements supportive for haemorrhagic control and wound healing, are most likely biocompatible with injured skin and could be described as hemostatic agents with an ability to form a mechanical barrier.

Witepsol is a typical industrial suppository base consisting of a mixture of mono-, di- and triglycerides. *Witepsol* is easy to handle; its melting procedure is facilitated by the non-overheating characteristic of the base. For this reason, *Witepsol* bases are widely used on the industrial scale for the preparation of pharmaceutical suppositories. Lidocaine hydrochloride, as an anesthetic, is commonly used in preparations such as suppositories, creams, gels and solutions. Solid paraffin (adjuvant) is commonly effective as a hardener and for the rise of the melting point of fatty base formulations. *Carbopol* (adjuvant) is a mucoadhesive material which is helpful for the enhancement of drug dissolution in biological environment.

Melting points of both formulations were in the 36.0–37.0° C temperature range; as this characteristic is critical for a suppository under physiological conditions. Mass uniformity is an important characteristic of single-dose preparations because it ensures (a) equal distribution of active and adjuvant ingredients and (b) the therapeutic window of a drug (the therapeutic window (or the pharmaceutical window) of a drug is the range of drug dosages which can treat a disease effectively without having toxic effects, author's note). The deviation (%) of suppositories weight was ±0.1, whereas the required deviation could be up to ±5% for n=20. Preparation of suppositories by molding is generally accepted as an effective and time-saving method in the pharmaceutical industry.

Drug release was performed in PBS at pH=6.8 at 37 °C with the aim to simulate the physiological pH of a rectum. As release of lidocaine hydrochloride into dissolution medium was slower for W-H35/CB-2 – a chitosan-enriched cuttlebone material – it proves the existing fact that chitosan has an impact on a controlled drug release. Other studies show the efficiency of chitosan as a drug reservoir, demonstrating the functionality of a chitosan/lidocaine system for the achievement of a prolonged anesthetic effect [51]. On the other hand, the drug release profile of rectal preparations does not fully reflect rectal absorption, and, therefore, the drug bioavailability. There are several physical-chemical factors affecting the rectal absorption, such as the drug solubility in a vehicle, the particle size, the nature of the base, the spreading capacity and other related physical factors. The release rate of lidocaine hydrochloride into the rectal fluid was expected to be high because its solubility in water is 50 mg/mL, while the pure drug is highly hydrophobic [52].

To conclude, chemical characterization of any hemostatic agents should be of exorbitant concern, because biocompatibility, especially hemocompatibility, is an essential measure to predict fortunate of any newly tested material. Elemental composition of any clotting agent is crucial despite any other characteristics, because it could arguably have an immediate impact on living tissue and further degree of success in wound healing. Cuttlebone fillers are characterized as multicomponent materials with

positive impact for bleeding control and wound healing. Cuttlebone fillers are supposed to be applicable to treat topical wounds directly or as a fillers for *Witepsol*-based suppositories to treat anorectal disorders with bleeding symptoms.

Conclusions

Bioinorganic elements of the cuttlebone, such as zinc, iron, calcium, magnesium and copper, are supportive for wound healing. Cuttlebone is aluminum-free, and therefore has an advancement over aluminosilicate hemostatic minerals as aluminum is associated with suppressed collagen synthesis. Modified, chitosan-enriched cuttlebone filler showed decrease in clotting time by 20%. Both cuttlebone fillers could be characterized as a hemocompatible, promising clotting agents with an ability to form a mechanical barrier for bleeding control. *Witepsol*-based suppositories with cuttlebone fillers have demonstrated favorable characteristics, such as melting point in a 36.0–37.0° range, dissolution time no longer than 30 min and gradual release of the anesthetic lidocaine hydrochloride. Based on the primal, though essential to conduct, *in vitro* test results, application of cuttlebone fillers could open a new page in the development of successful naturally-based local hemostatic and wound healing products.

Abbreviations

CB-1	Original cuttlebone material
CB-2	Modified cuttlebone material
RBC	Red blood cells
SEM	Scanning electron microscopy
SEM-EDX	Scanning Electron Microscopy with Energy Dispersive X-Ray
PBS	Phosphate buffer solution
W-H35	*Witepsol* H-35
W-H35/CB-1	*Witepsol* H-35 with CB-1 filler
W-H35/CB-2	*Witepsol* H-35 with CB-2 filler

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Essential data generated or analyzed during this study are included in this published article additional file. Any other aditional datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The author declare that they have no competing interests

Funding

No funding information to declare

Author's contribution

The author by herself has designed the work, its analysis, and made data interpretation. The article is based on the author's doctoral dissertation "Characterization and Application of Cuttlebone for Development of Biomedical and Pharmaceutical Compositions", 2018, ISBN (978-609-02-1454-1) and partly on the oral presentation "Cuttlebone as a Blood-Stopping Agent" at XI International War and Extremal Medical Conference, Kaunas, 17th of May, 2018. However, this article is strongly improved to reveal the topic in detail.

Acknowledgments

This manuscript is written with gratefulness and love to my (author's) father, the great sailor and voyager. The author shows her appreciation to the military doctor Lieutenant Colonel Gintaras Berkevičius from Lithuanian Armed Forces Defense Staff, Vilnius, Lithuania, for valuable pieces of advice regarding the topic. The author thanks Dr. Sigita Petraitytė, Dr. Monika Budvytienė and Dr. Sandra Kiseliovienė from Kaunas University of Technology, Lithuania, for technical support. The author is grateful to Mr. Guillermo Gonzales (USA) for English proofreading.

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October 2020.

Tables

Table 1. Elemental composition of cuttlebone fillers in comparison to mineral- and polymer-based materials from this study and reference data

Name	Element, %								
	Na	K	Zn	Fe	Cu	Si	Mg	Ca	Al
CB-1	0.83	0.13	0.12	0.02	0.005	–	0.17	50.70	–
CB-2	0.17	–	0.03	0.02	0.006	0.05	0.33	50.90	–
Celox	0.040	–	–	0.06	0.008	0.05	–	0.16	–
Bentonite	0.38	1.72	0.02	2.63	0.009	19.30	0.81	1.89	5.25
Talc	–	0.02	0.01	0.63	0.007	18.30	9.86	0.70	0.14
$CaCO_3$	–	–	–	0.01	–	0.014	0.10	56.80	–
Zeolite [20]	1.41	2.27	–	0.68	–	–	0.11	1.93	6.10
QuikClot [20]	0.57	–	–	–	–	–	–	11.43	16.11

Figures

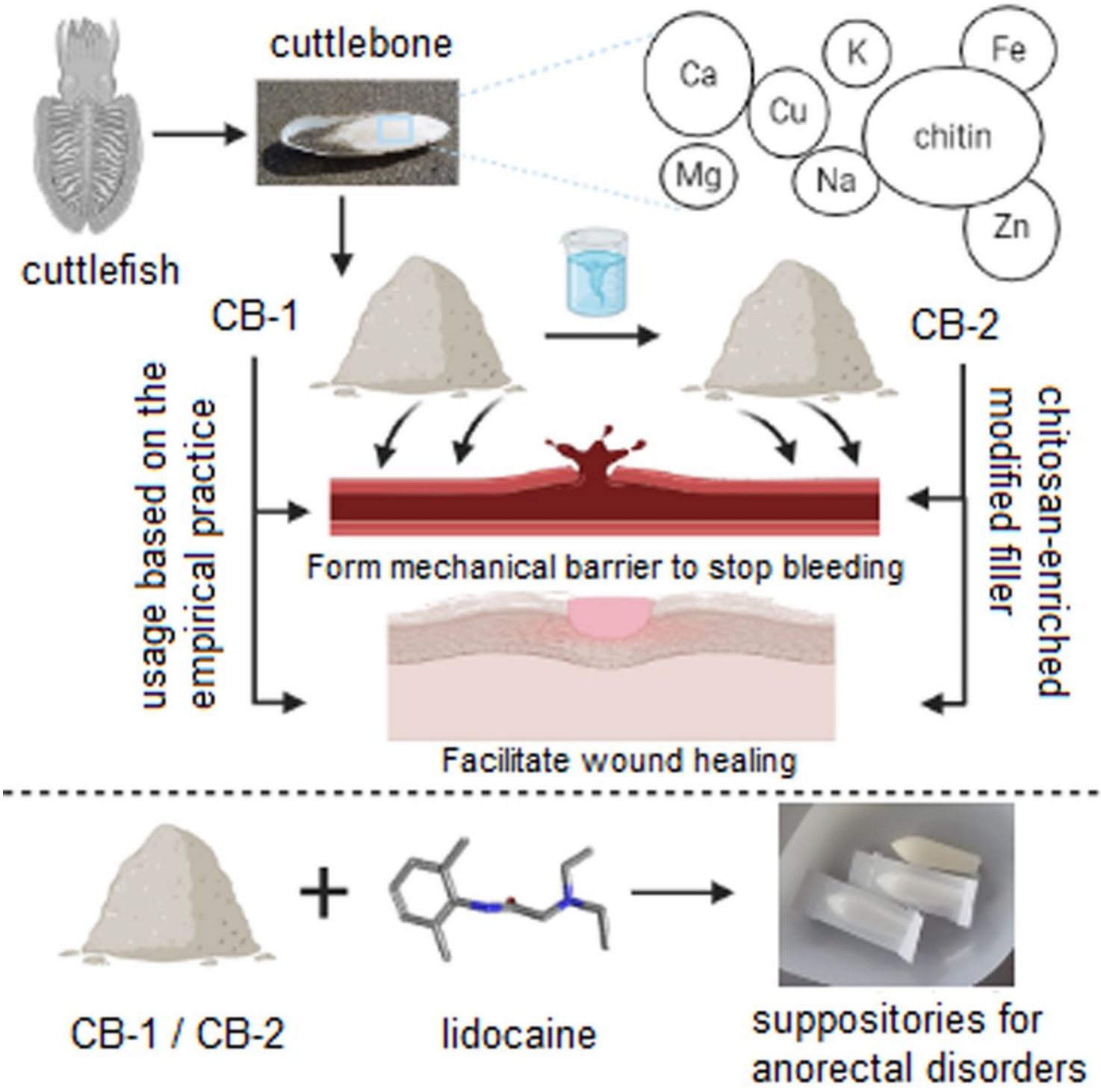
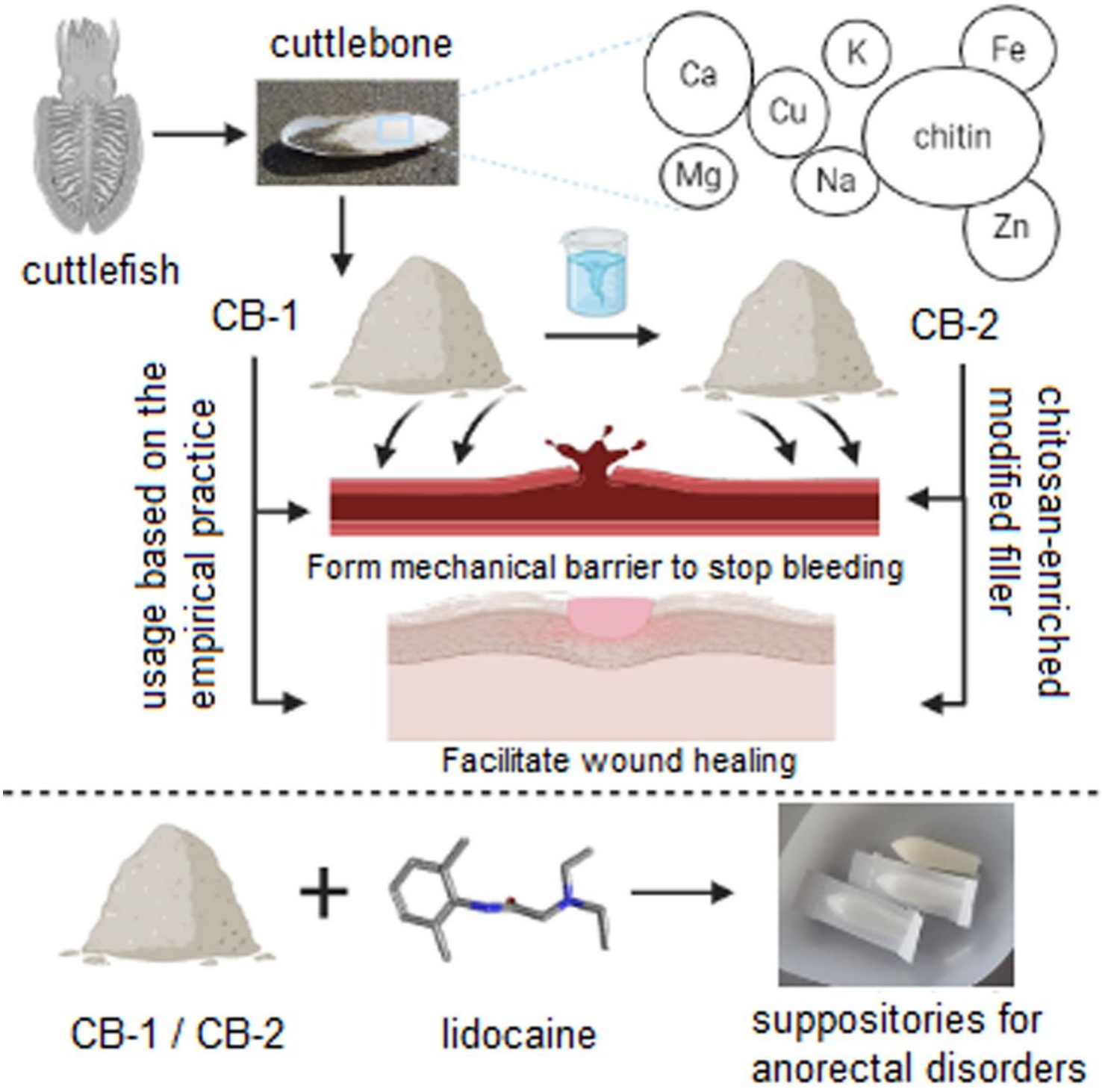


Figure 1

Summary graphic illustration describing preparation and characteristics of cuttlebone fillers and further preparation of lipophilic suppositories for anorectal disorders



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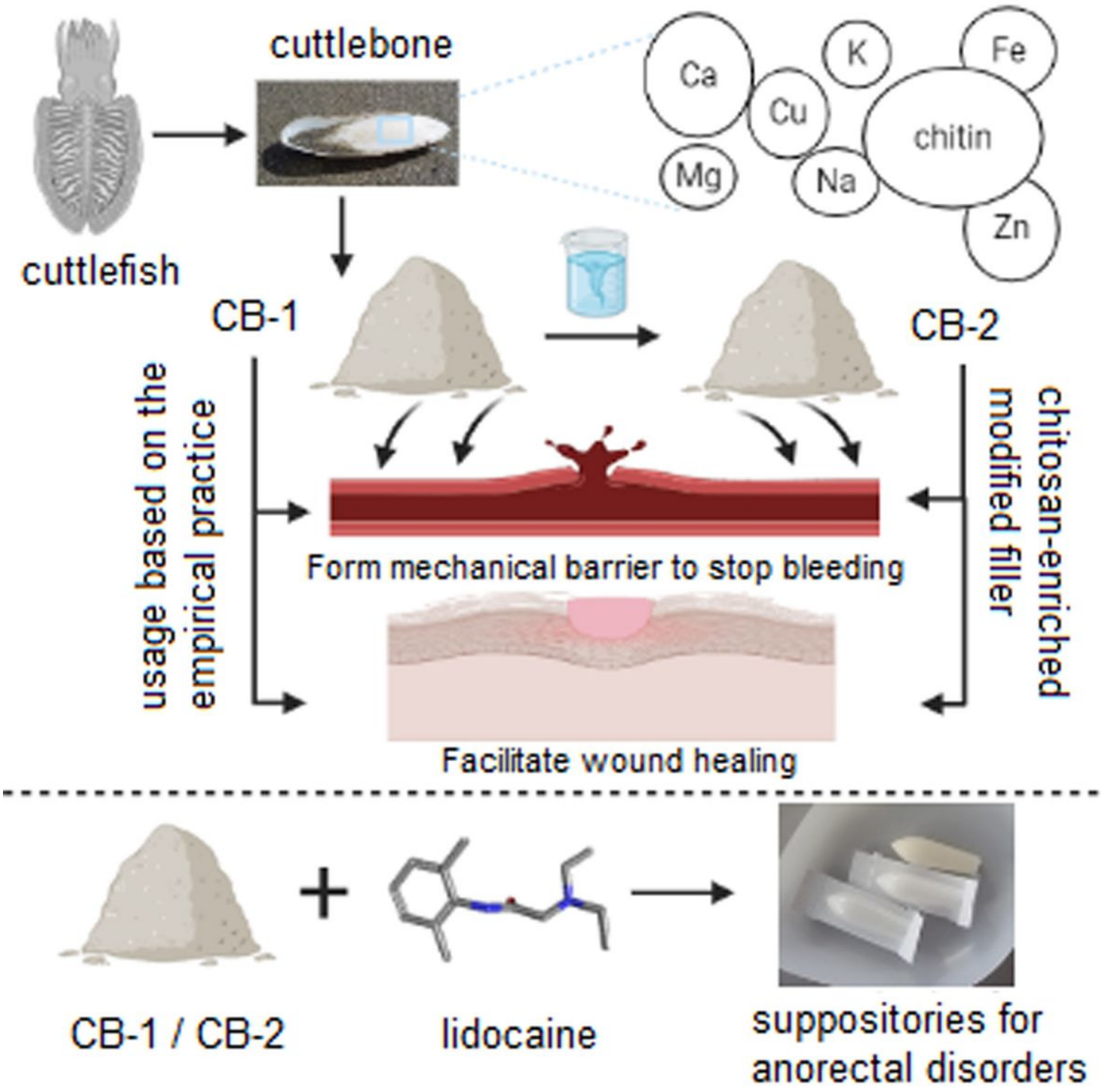

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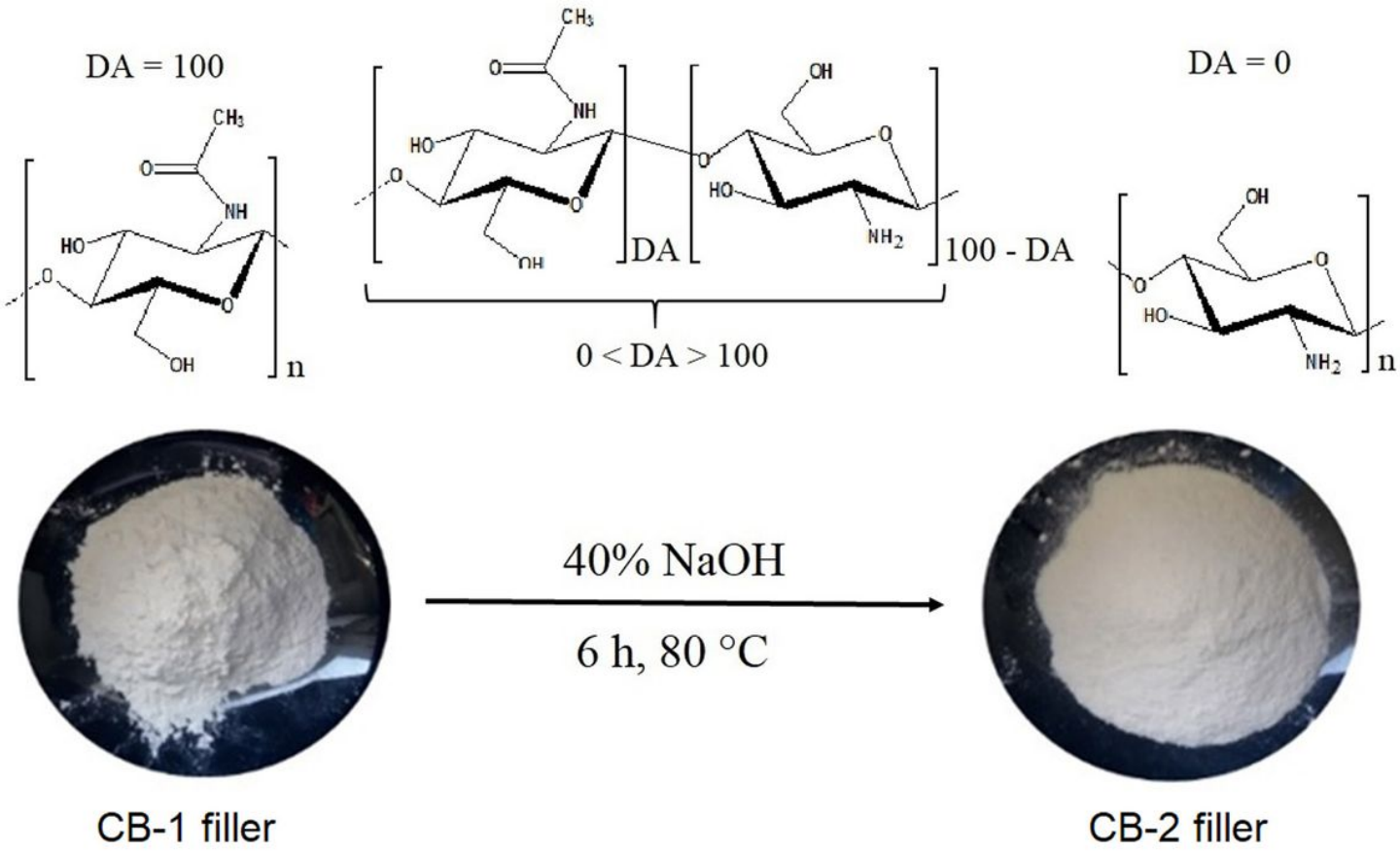
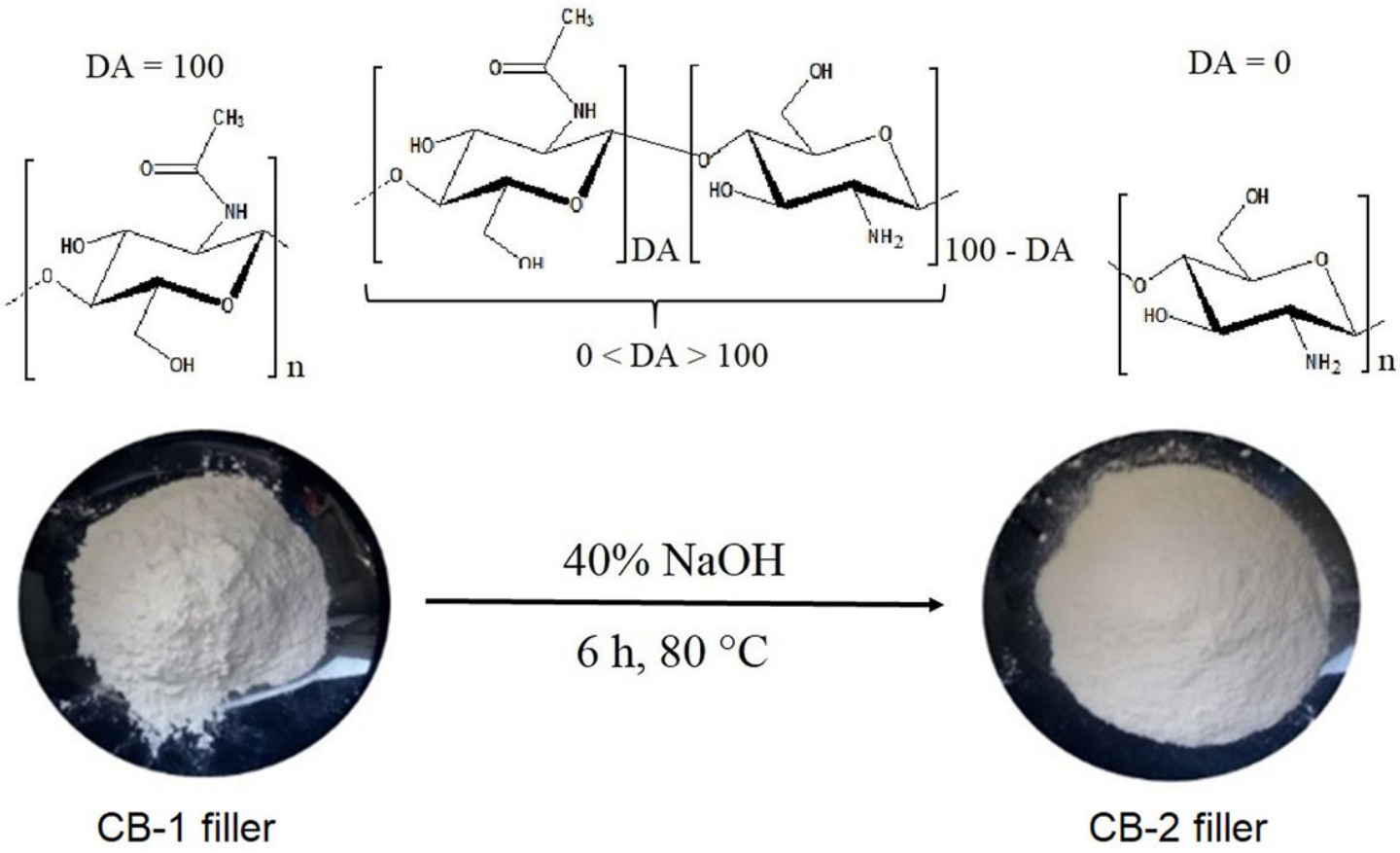


Figure 2

Preparation of cuttlebone fillers CB-1 (non-modified) and CB-2 (alkali-treated). Fragments of chitin and chitosan structure are presented, DA is a degree of N-acetylation



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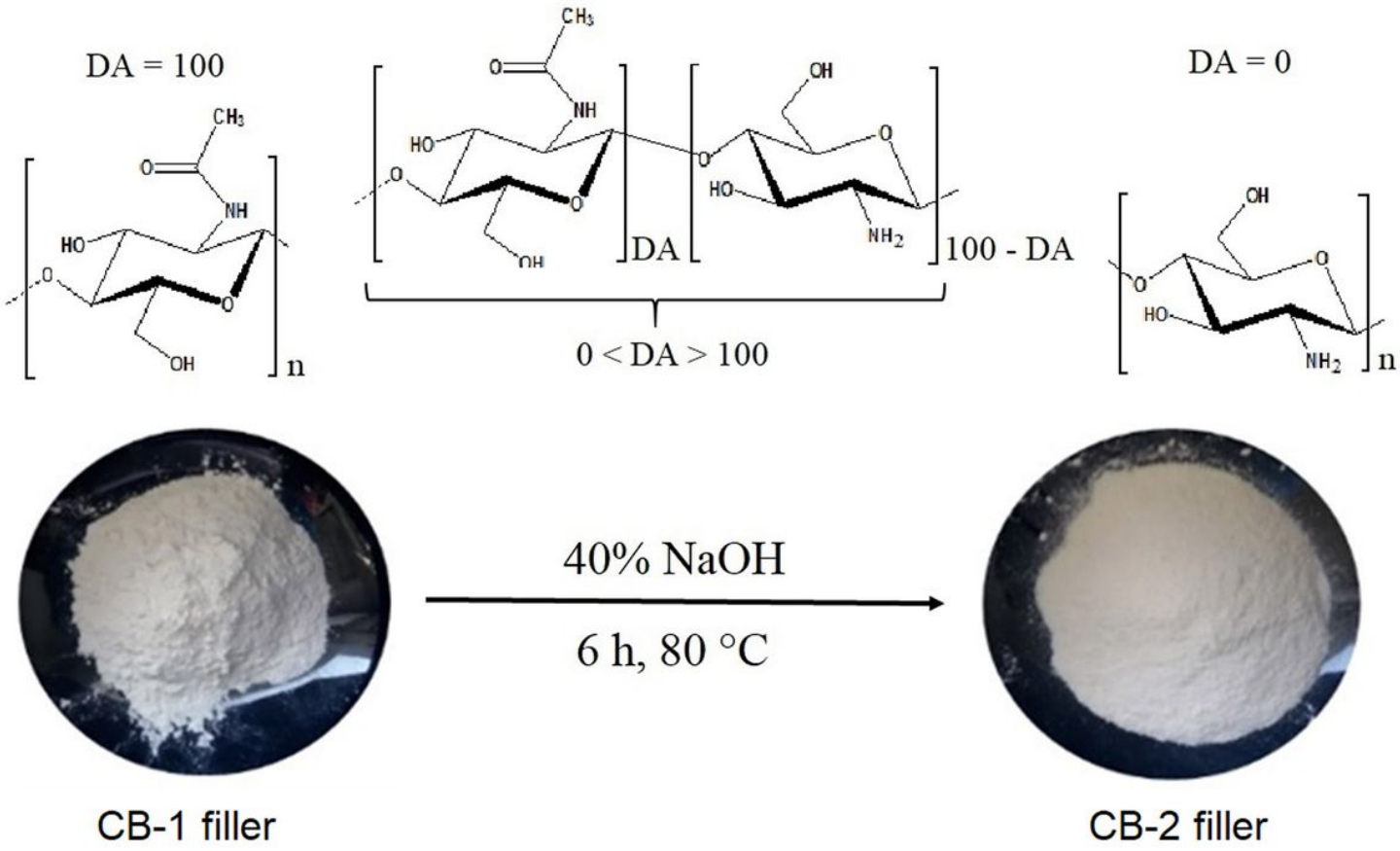

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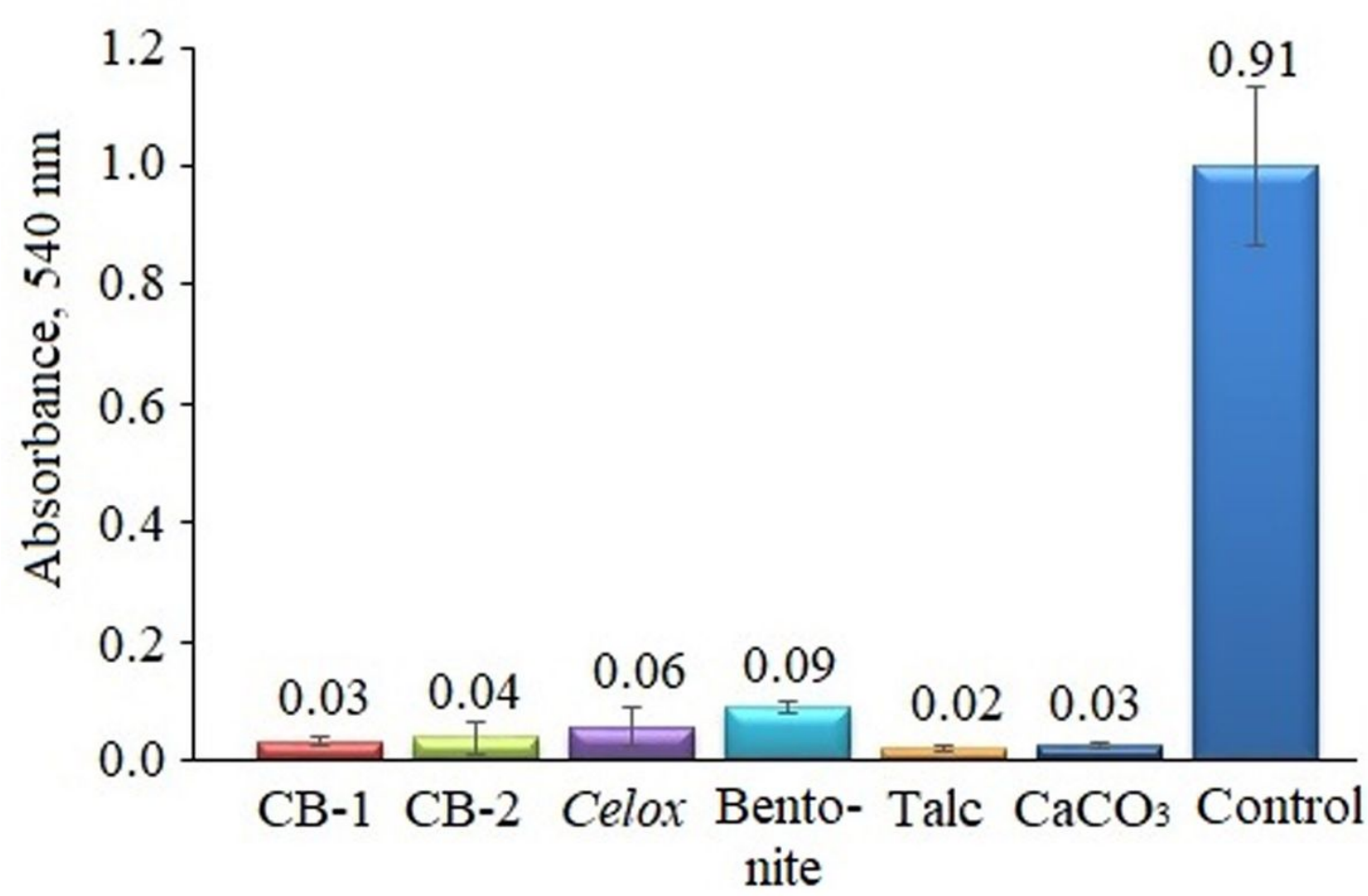
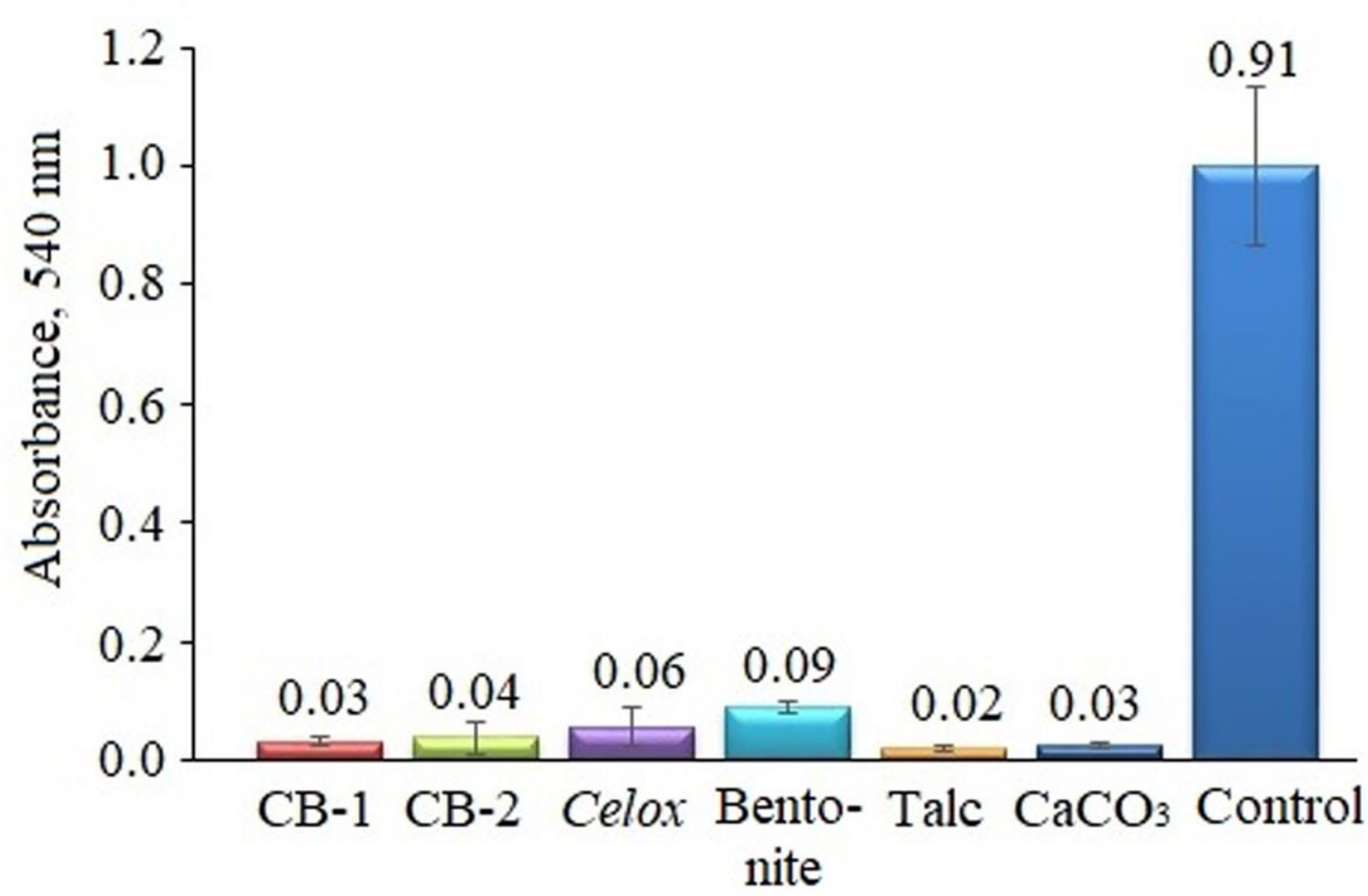


Figure 3

Blood clotting test: hemoglobin absorbance of cuttlebone fillers in comparison to other materials



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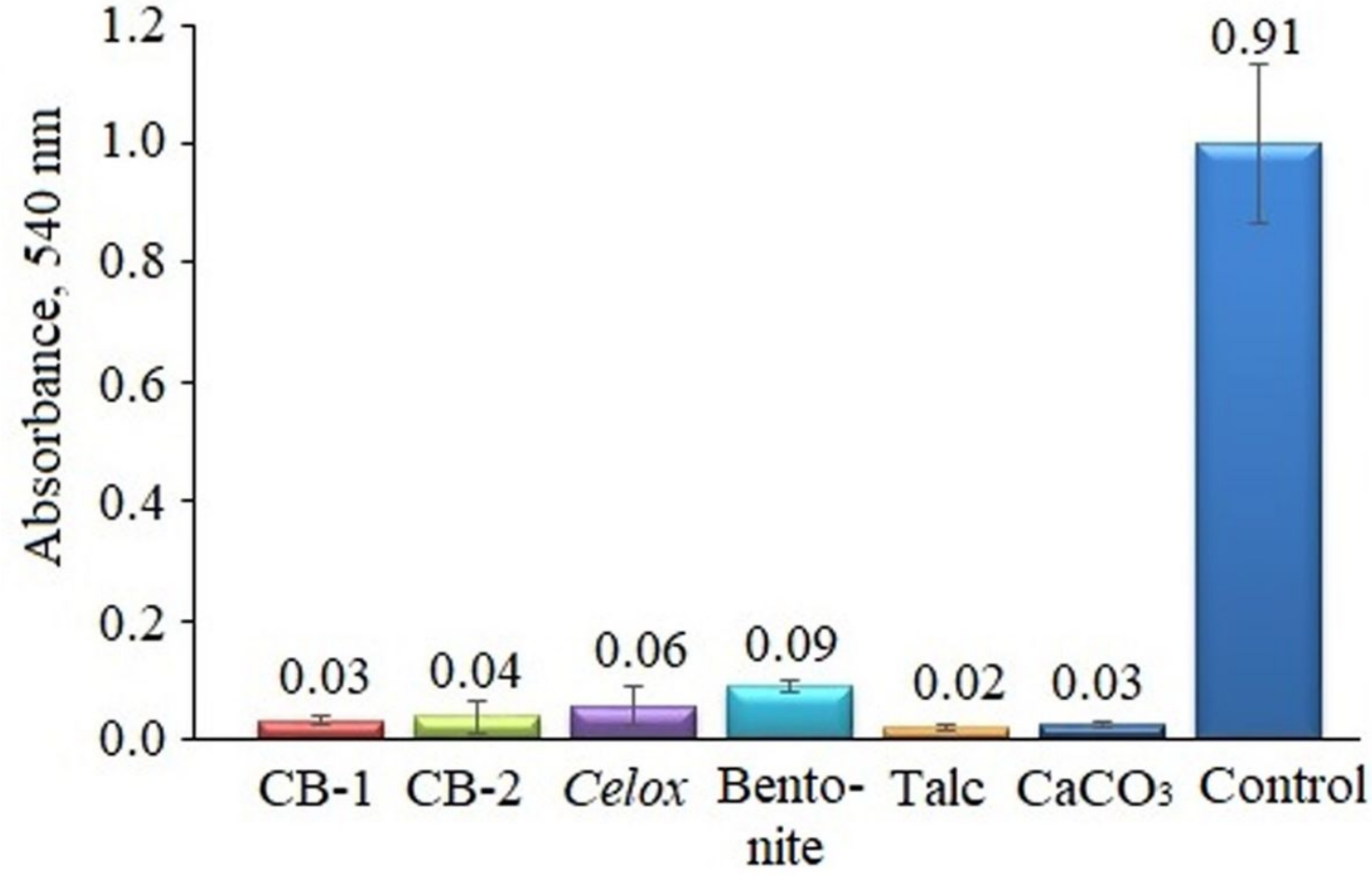

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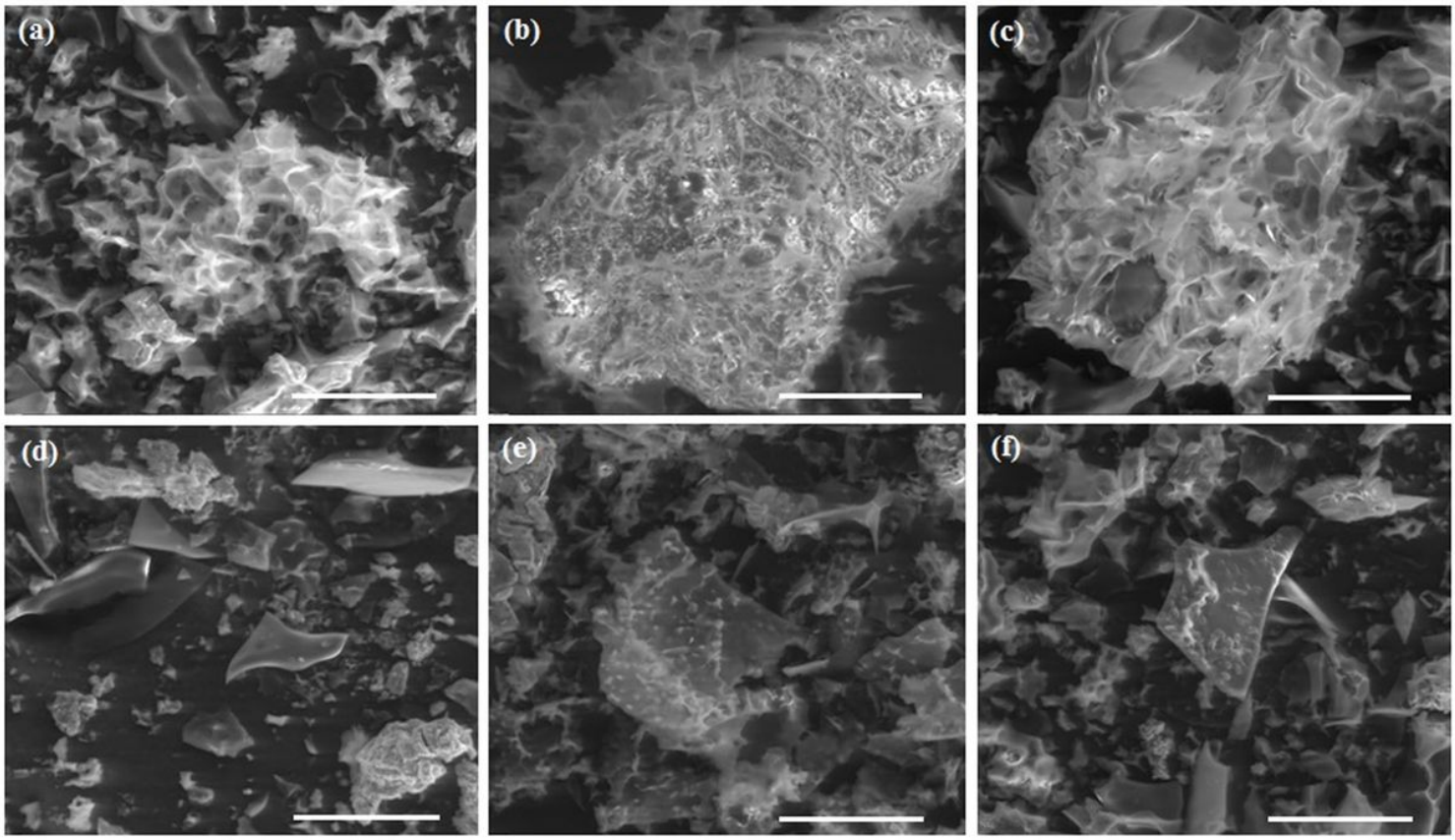
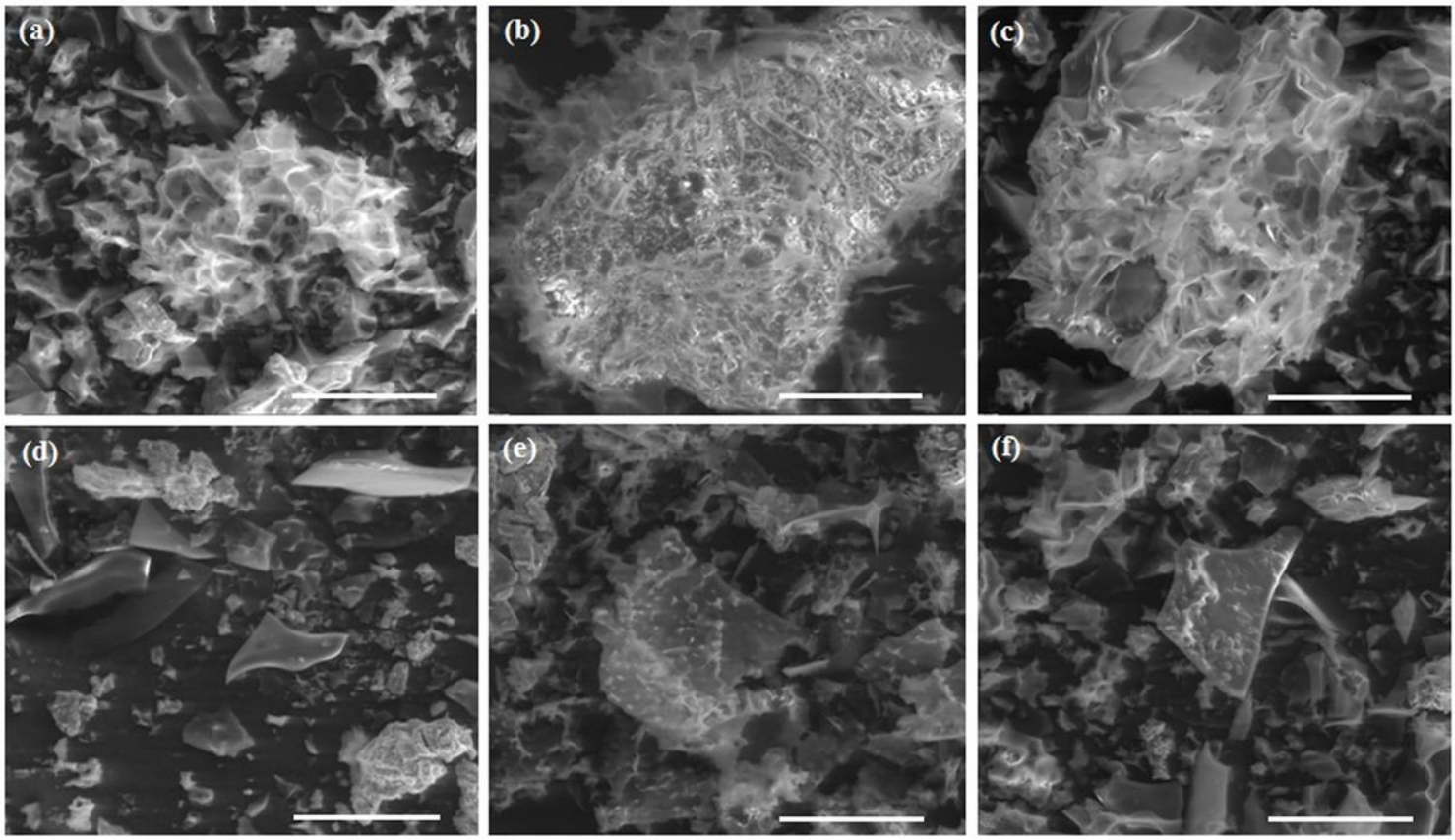


Figure 4

Erythrocyte shapes in cuttlebone and other powdered materials after clotting compared with a control sample (whole blood): (a) CB-1, (b) CB-2, (c) Celox, (d) bentonite, (e) talc and (f) calcium carbonate. Circles indicate a zone of erythrocytes. Magnification x2500, scale bar = 40 μm



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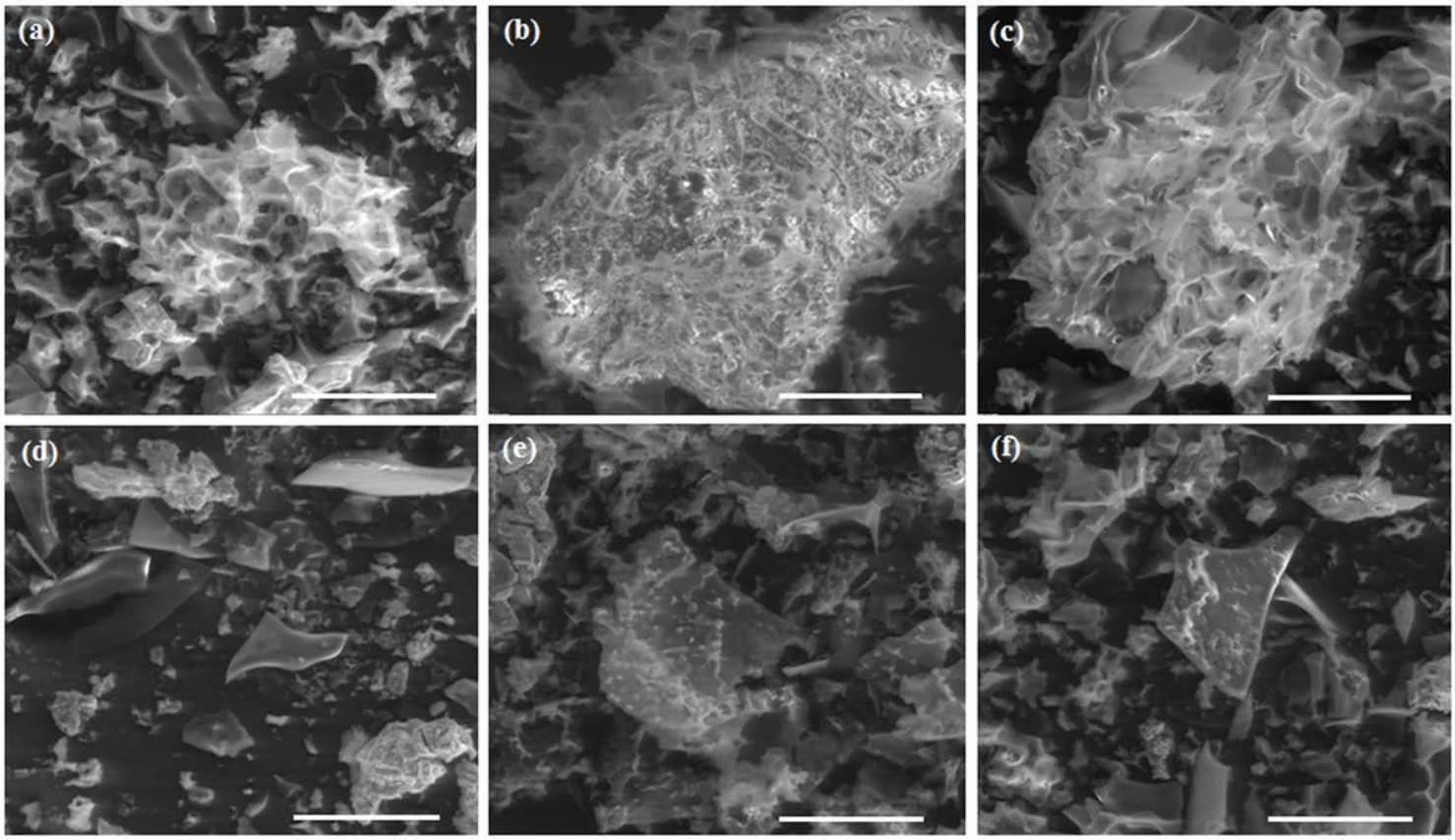

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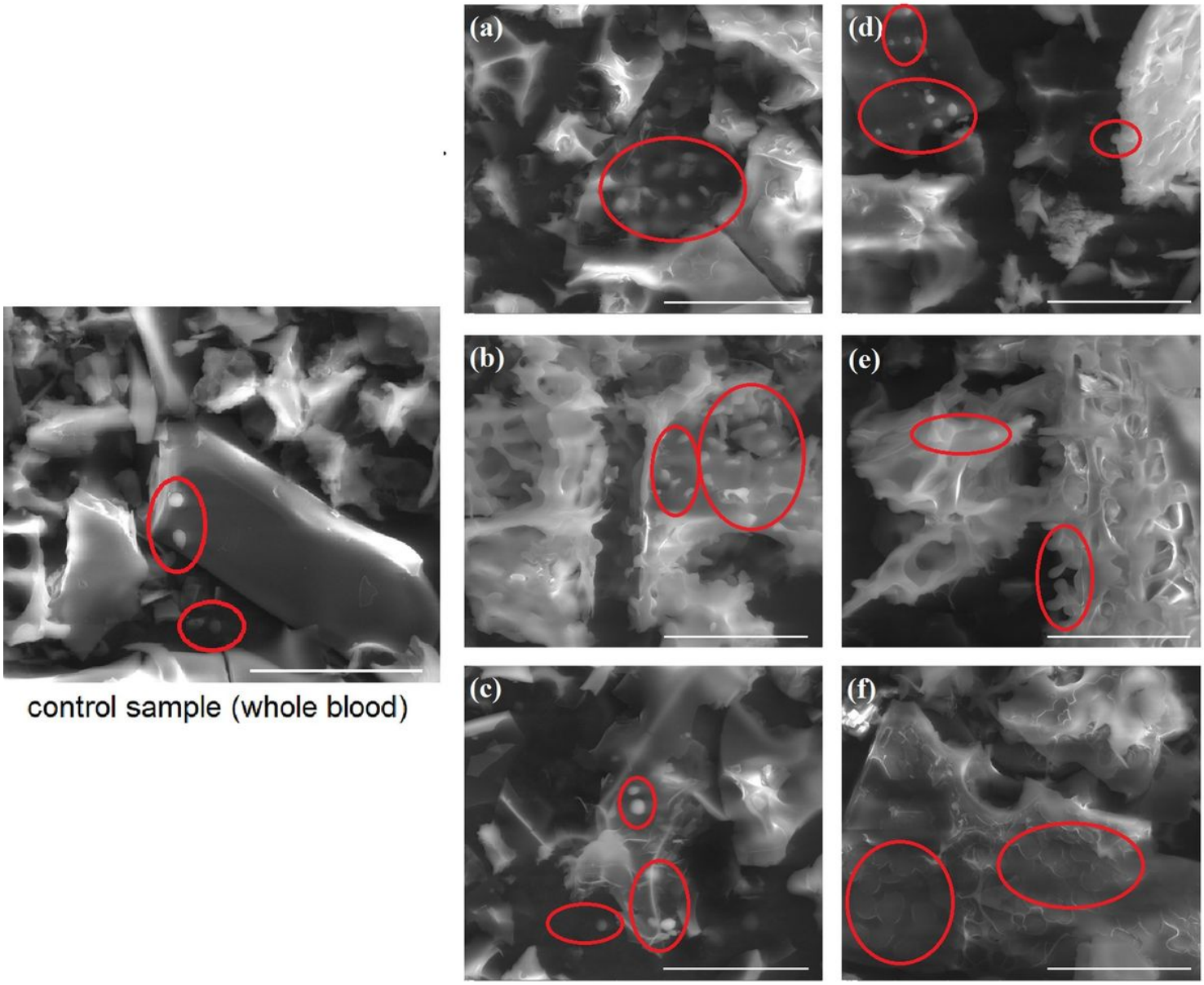
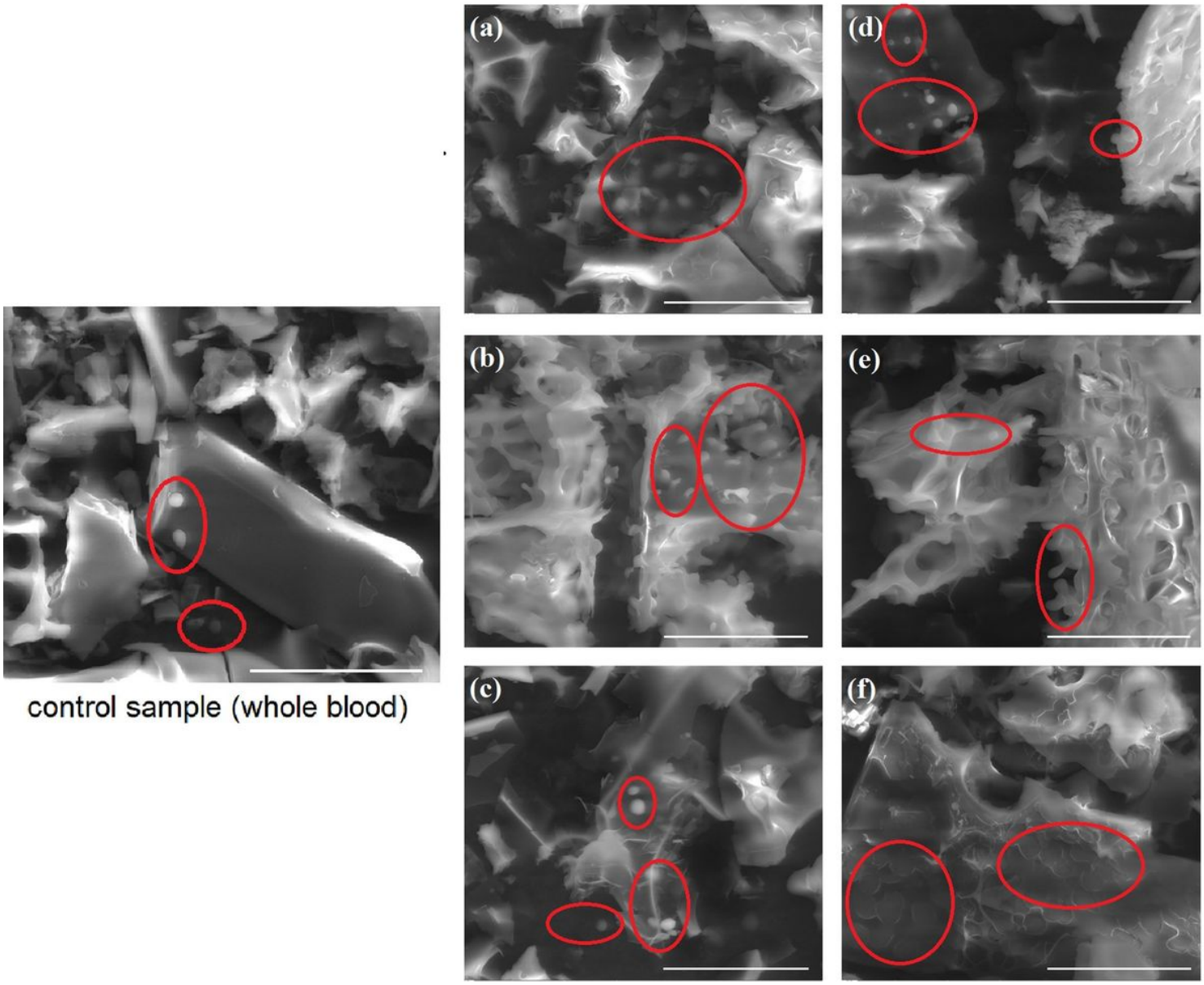


Figure 5

Microphotographs of clotted blood after interaction with cuttlebone fillers and other powdered materials, surface view: (a) CB-1, (b) CB-2, (c) Celox, (d) bentonite, (e) talc and (f) calcium carbonate. Magnification x750, scale bar = 100 μm



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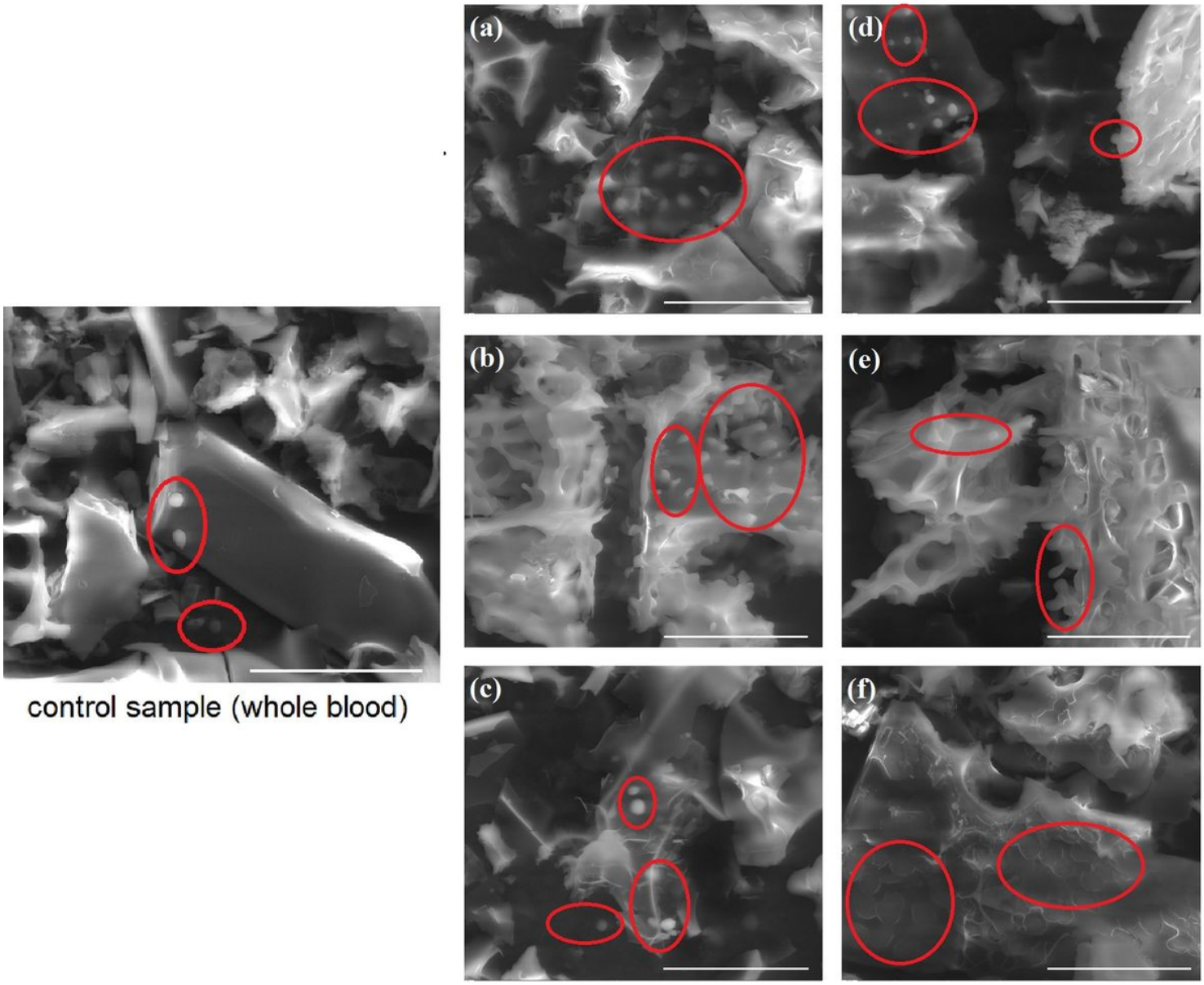

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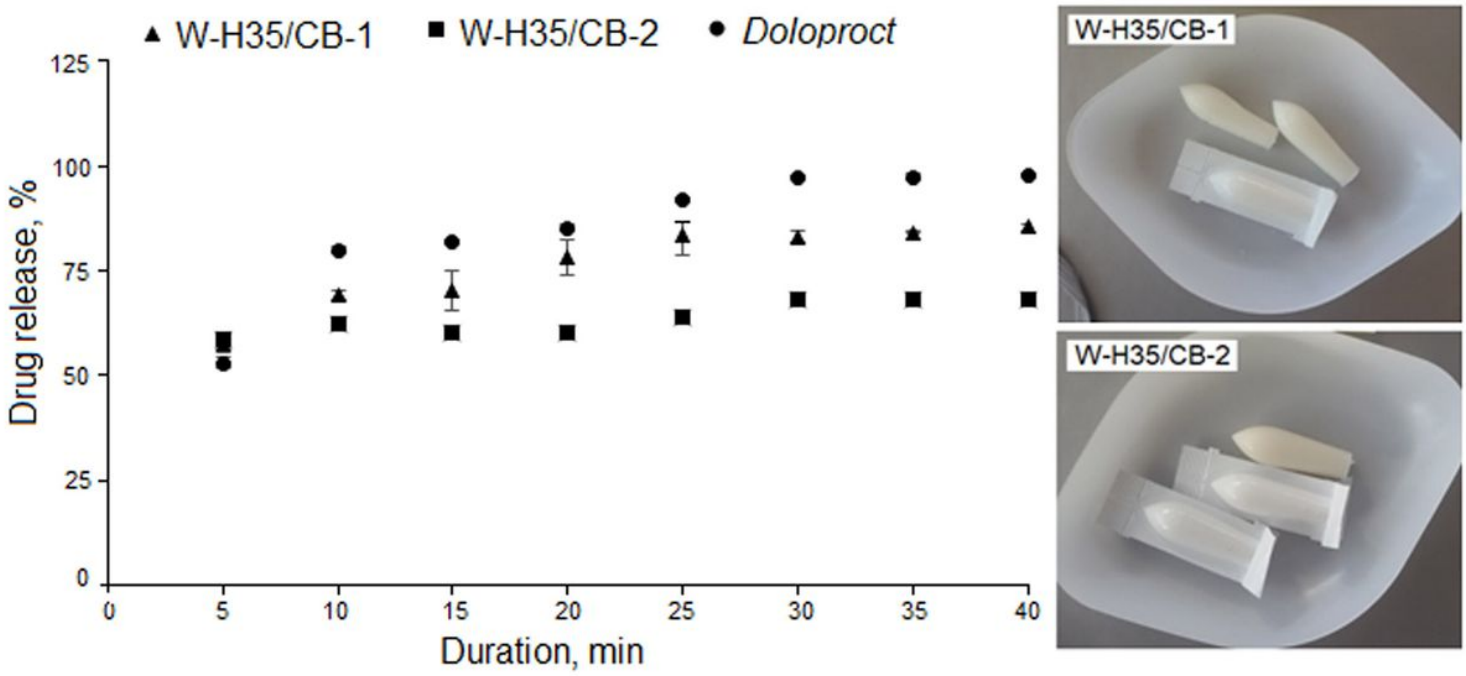
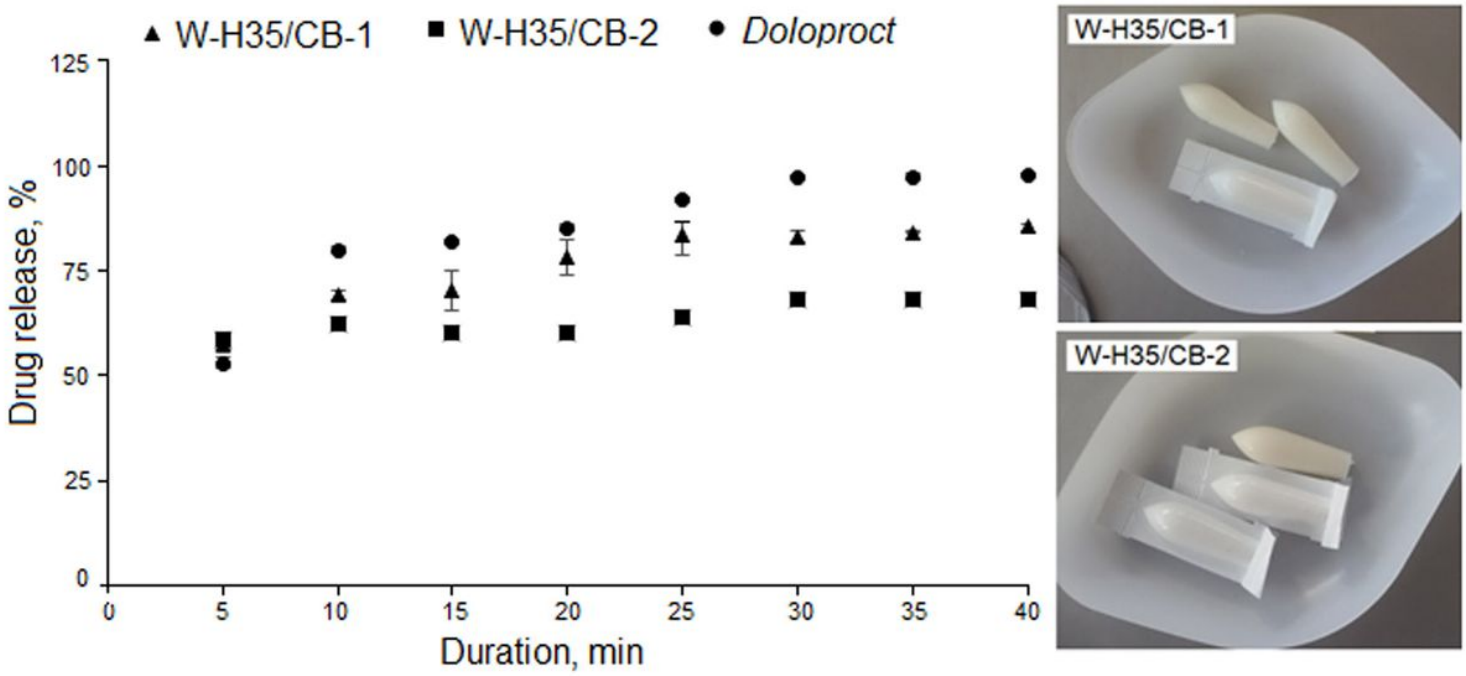


Figure 6

Lidocaine hydrochloride release from Witepsol-based suppositories with cuttlebone fillers



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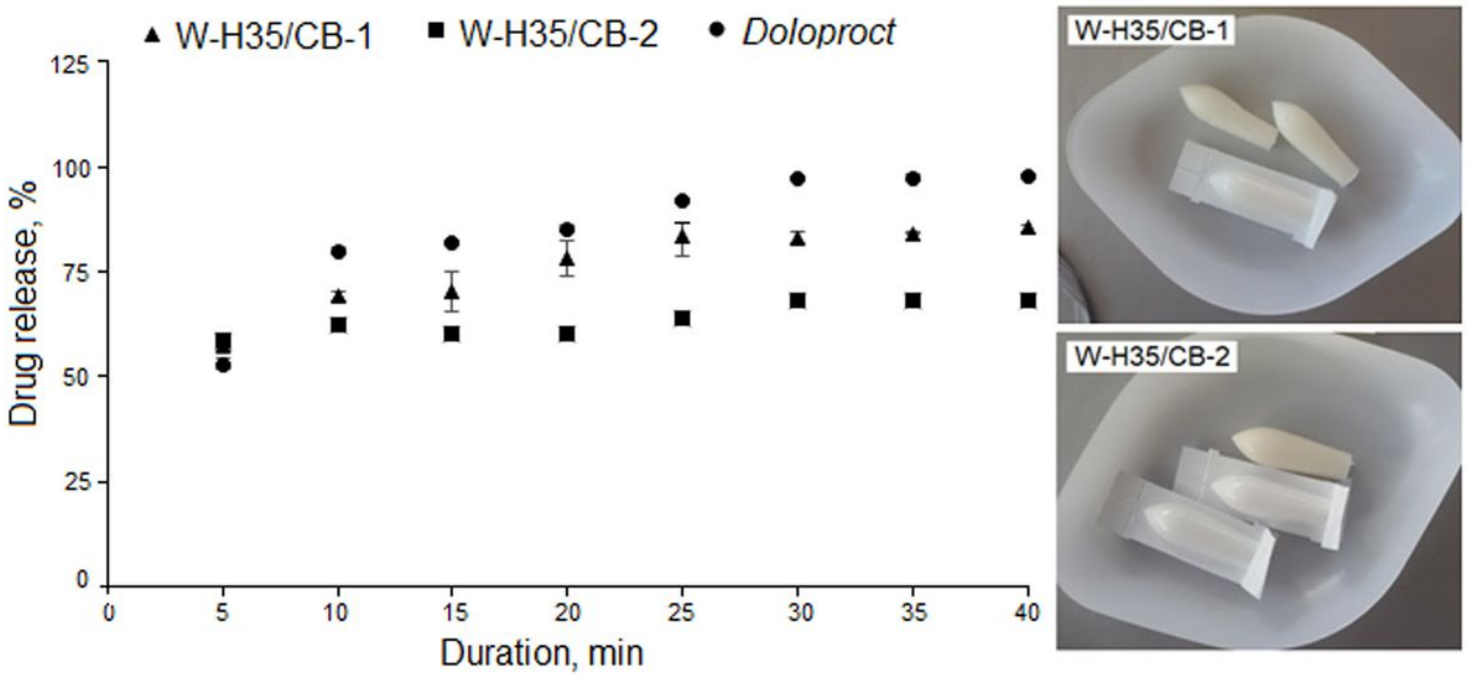

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Lidocaine hydrochloride release from Witepsol-based suppositories with cuttlebone fillers

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