ORIGINAL RESEARCH

Can an Alginate-based Wound Dressing Modified with Garden Cress Substitute for COE-PAK as a Wound Dressing? An *In Vitro* Study

Nahla Abdelmonem[1], Rania Salama[2], Dina H Mostafa[3]

Abstract

Aim: The aim of the current study was to prepare a natural oral wound dressing from alginate modified with garden cress (GC), a rich source of antibacterial phytochemical compounds essential for wound healing.

Materials and methods: Sodium alginate (SA) dressing (negative control group), was prepared and modified with GC seeds extracts (25 µg/mL and 50 µg/mL) as the intervention groups, and COE-PAK was the positive control group. Cytotoxicity was measured using WST-1 assay ($n = 15$) after 24 and 48 hours. The *in vitro* wound healing assay ($n = 15$) was assessed in terms of wound width, and cell migration rate (0, 24, 48, and 72 hours). Agar diffusion test was performed to investigate the antibacterial action ($n = 15$) of the groups against *Streptococcus mutans* and *Lactobacillus casei* strains. Results were significant at $p \leq 0.05$.

Results: There was no statistically significant difference in cytotoxicity in all groups ($p = 0.24$ at 24 hours and 0.1 at 48 hours). Garden cress-containing groups revealed the lowest mean value of wound width (0.27 mm ± 0.01 and 0.23 mm ± 0.01 for 25 µg/mL and 50 µg/mL, respectively at 48 hours) and the highest mean value of cell migration rate (0.013 mm/hour ± 0.004 and 0.014 mm/hour ± 0.004 for 25 µg/mL and 50 µg/mL, respectively at 48 hours), in addition to the highest antibacterial action (1.49 mm ± 0.05 and 2.14 mm ± 0.09 for 25 µg/mL and 50 µg/mL, respectively against *S. mutans*, 1.43 mm ± 0.07 and 2.55 mm ± 0.09 for 25 µg/mL and 50 µg/mL, respectively against *L. casei*).

Conclusion: Alginate wound dressing modified with GC extract could be considered a promising wound dressing material in terms of wound healing and antibacterial action.

Clinical significance: Ready-to-use alginate-based wound dressing modified with GC extract may represent a promising natural alternative to the most commonly used oral wound dressing (COE-PAK).

Keywords: Alginate, Antibacterial, COE-PAK, Cytotoxicity, Garden cress, *In vitro* wound healing.

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Introduction

Isolation of the surgical wound from the oral environment may be necessary in order to protect the surgical area, allow faster healing and provide patient comfort.[1] This may be achieved via the application of a wound dressing. A wound dressing is any material that is used in direct contact with the wound to promote healing and prevent further complications. An ideal wound dressing should be biocompatible and should not induce any allergic reaction. It should also possess an antibacterial effect to prevent bacterial colonization, to stabilize free gingival grafts; and should have an acceptable taste with a reasonable setting time.[2]

The history of wound care can be traced to the ancient Egyptian civilization, 1600 BC. Recently, wound dressings are classified as: passive, interactive, and bioactive based on their action. Traditional dressings as gauze are passive products. Interactive products include polymeric films and foams, which are almost transparent, permeable to water vapor and oxygen but impermeable to bacteria. Bioactive dressings deliver substances active in wound healing. These materials include proteoglycans, collagen, chitosan, and alginates.[2]

In the oral cavity, dental plaque is considered a chief predisposing factor for delayed wound healing. Many studies have investigated the microbiology of dental plaque that colonizes the surgical sutures. It was revealed that *Streptococcus mutans* and *Lactobacilli* are the main population of dental plaque.[3,4] Intraoral wound dressings are commonly classified according to the presence of eugenol into either zinc oxide eugenol or zinc oxide non-eugenol dressings. The first intraoral wound dressing introduced was "Wonder Pack" by Aw Ward in 1923 which was based on zinc oxide eugenol.[5] Zinc oxide eugenol dressings have been used for several years in the oral cavity. However, owing to the cytotoxicity, tissue irritation, spicy taste, and stiff cement-like consistency of zinc oxide eugenol dressing, it is no longer used nowadays and was substituted by non-eugenol dressings.

COE-PAK was introduced more than 50 years ago by (COE Laboratories Inc., Chicago, IL, USA). It is one of the most commonly used non-eugenol periodontal pack and oral wound dressing.[6] It is supplied as either a two-paste system or an auto-mixing

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paste in a syringe. The base tube contains oils of clove (principal ingredient), rosin (plasticizer), fatty acids (lubricating agent), zinc acetate (strengthening agent), chlorothymol (bacteriostatic agent), and ethyl alcohol (control viscosity). The accelerator tube contains zinc oxide and magnesium oxide (principal ingredients), vegetable oil (plasticizer), chlorothymol, lorothidol (fungicide), silica (filler) and coumarin as anticoagulant.[7] Once the two pastes are mixed, a chemical reaction takes place between zinc oxide and magnesium oxide on one side and fatty acid on the other side leading to setting of the material. Based on several studies, it was reported that COE-PAK does not induce burning sensation, provides close adaptation to teeth and tissue to protect the wound, and does not cause any unpleasant taste or odor. However, results revealed that it is cytotoxic with different degrees and has questionable antibacterial action.[7–9]

Alginate hydrogels have been also used in wound healing and drug delivery. Alginate is a natural, biocompatible, non-branched polysaccharide obtained from brown seaweed with hydrocolloid properties. It is composed of monomers of β-D-mannuronic (M blocks) and α-L-guluronic acids (G blocks). The carboxyl group in alginates is responsible for the mucosal adhesion characteristic of alginate.[10]

Although alginate has been proven to have several advantages as a wound dressing material, it still has limited antibacterial action and limited ability to promote healing.[11,12] Hence, several studies were conducted to improve alginate properties. Nanosilver,[13] ginseng extracts,[14] honey,[15] and *Satureja khuzistanica* leaves extract,[16] were added to alginate in an attempt to improve its antibacterial action or healing potential. Unfortunately, none of the investigated additives could provide both.

Garden cress (GC) (*Lepidium sativum*) is an annual, herbaceous edible plant, botanically related to mustard and watercress. Garden cress seeds are small, reddish brown in color, oval in shape, with a minor wing-like extension present on both the edges of the seed.[17] Several studies revealed the role of GC in the protection of cell against cytotoxic agents,[8,9] boosting the immune system,[18,19] inhibition of cancer cells[20,21] and antioxidative action.[22,23] Owing to their flavonoid content, GC extracts were proven to have both anti-inflammatory and pain-reducing properties.[24] Alobaidi evaluated the anticancer activity of GC leaf on human tongue squamous cell carcinoma (CAL-27). The results revealed inhibition in the growth of cancer cells, in a dose-dependent manner.[25] In addition, GC was proposed for use in the treatment of chronic periodontitis. Shawki et al. evaluated the effect of GC on chronic periodontitis in postmenopausal osteoporotic women. Garden cress group showed a significant decrease in the mean gingival index and pocket depth compared with local debridement group.[26]

Therefore, the aim of the current study was to prepare and evaluate a novel alginate-based wound dressing film containing ethanolic extract of GC seeds regarding its cytotoxicity, wound healing ability and antibacterial action compared with COE-PAK dressing. The null hypothesis was that there will be no difference in the cytotoxicity of the GC seed ethanolic extract incorporated in sodium alginate (SA) film compared with COE-PAK dressing.

Materials and Methods

This *in vitro* study was conducted in the Biomaterials Department, Faculty of Dentistry, Cairo University, Egypt. The study period was from January 1, 2023 to July 1, 2023 after approval of the Dental Research Ethics Committee No. 191112, Faculty of Dentistry, Cairo University, Egypt.

Sample Size Calculation

Sample size calculation was performed using PS Power and Sample Size Calculations software, version 3.0.11 for MS Windows (William D. Dupont and Walton D., Vanderbilt University, Nashville, Tennessee, USA). Kadkhodazadeh M et al. reported that the mean ± SD of percent of cellular viability in GC seeds extract group (24 hours) was approximately 92.5% ± 4.8, while in COE-PAK group, it was approximately 11.5% ± 0.7. Based on both studies using Student's *t*-test, the sample size that would detect a difference in cellular viability of 5% with 80% power at $\alpha = 0.05$ level was equal to 15.[27]

Study Design and Grouping

The specimens were assigned into four groups according to the intervention. The negative control group was the prepared SA dressing, the positive control group was COE-PAK, and the intervention groups were GC seed ethanolic extract (25 µg/mL and 50 µg/mL) added to SA colloidal solution dressings, abbreviated as 25 GC and 50 GC, respectively.

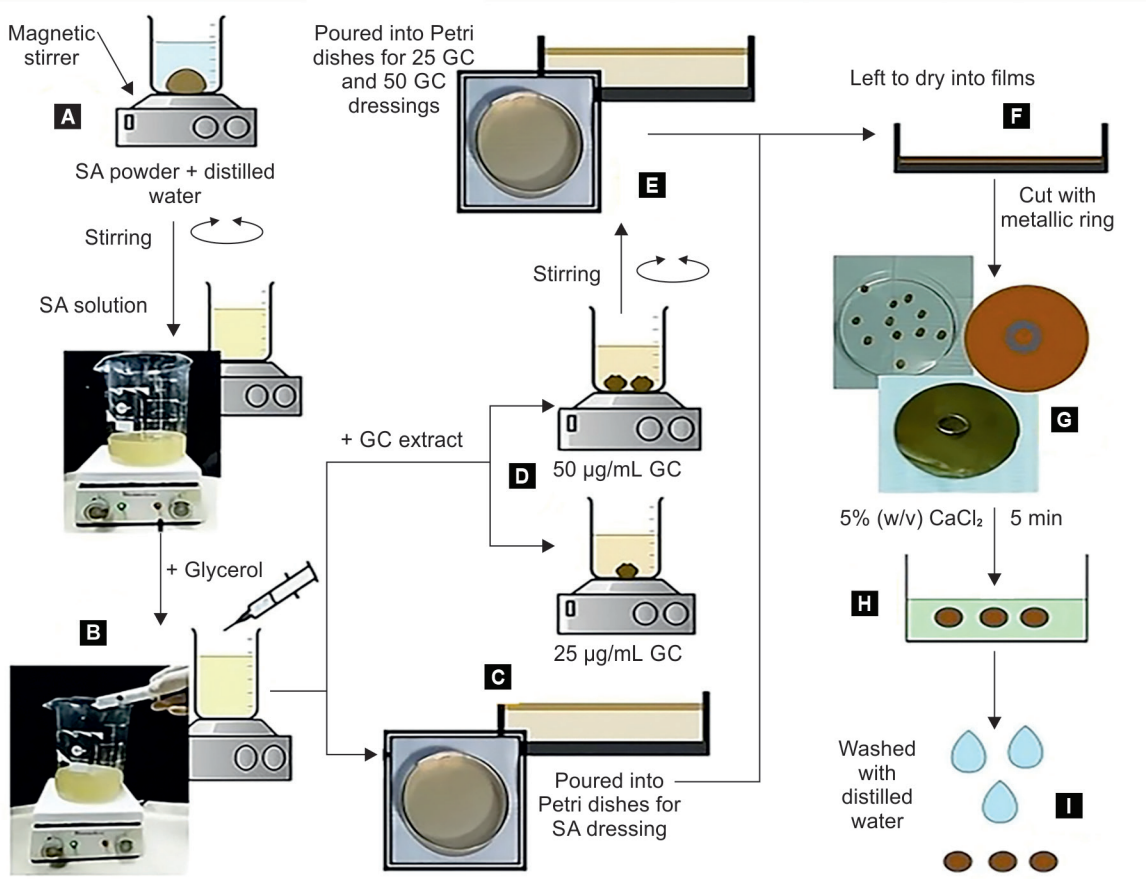
Preparation of Garden Cress Seeds Ethanolic Extract

Garden cress seeds were purchased from SHANA Company. A voucher specimen (no. 16112020) was deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy. Seeds were ground into powder using an electric milling machine with 4000 rpm for 4 minutes.[28] The extracts were prepared using the "Cold Simple Maceration" technique described by Rafińska et al. Three hundred grams of the GC powder was weighed using an electronic sensitive balance (HR200, A&D, USA), and were added to 3 liters of 95% ethyl alcohol. The mixture was left for 2 days at room temperature (24°C) with intermittent stirring. The process was repeated twice using fresh solvent until clear colloidal solution (indicating complete extraction) was obtained. Following extraction, the mixture was placed in a rotary evaporator machine (temperature: 55°C; pressure 175 mbar) to yield a semisolid mass. The obtained extract was stored at –20°C.[28]

Chemical Characterization of the Prepared Garden Cress Seeds Ethanolic Extract

Gas Chromatograph with Mass Spectrometer (GC/MS) Analysis

The prepared extract was analyzed using gas chromatograph coupled with mass spectrometer (ISQ Single Quadrupole MS, Thermo Scientific, USA). An electron ionization system with ionization energy of 70 eV was used. Helium gas was utilized as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperature was set at 280°C. The oven temperature program was set at an initial temperature of 50°C for 2 min. This was followed by a 5°C/min increase until the temperature reached 270°C. Finally, the oven temperature was raised to reach 310°C. All identified components were quantified with reference to investigate a percent relative peak area. Identification of the components was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.[28]



Figs 1A to I: A schematic diagram representing the steps of the experimentally prepared hydrogel dressings' specimens. (A) SA powder was added to water by 2% (w/v) and stirring was performed with magnetic stirrer; (B) Addition of glycerol (1% v/v); (C) The solution was poured into Petri dishes to form SA dressing; (D) Addition of (25 µg/mL or 50 µg/mL) ethanolic extract of GC seeds to the film-forming solution of SA to prepare 25 GC and 50 GC solutions, respectively; (E) 25 GC and 50 GC solutions were poured into Petri dishes; (F) Petri dishes containing dressings colloidal solutions were left to dry into films; (G) A metallic ring (5 × 2 mm) was used to cut films into specimens; (H) Specimens immersed into 5% w/v $CaCl_2$ solution for 5 minutes; (I) Washing specimens thoroughly with distilled water for 3 minutes

Determination of the Phenols Content

The extract was weighed using an electronic sensitive balance and was mixed with 1 mL of Folin Ciocalteu reagent. After 3 minutes, 1 mL of saturated sodium carbonate solution was added, and mixed well, followed by the addition of 3 mL of distilled water. The reaction mixture was kept in a dark place for 1 hour with intermittent shaking. The absorbance was measured at 725 nm using a spectrophotometer (Jasco V-630 spectrophotometer, Japan). Phenolic contents were calculated on the basis of the standard curve for gallic acid. The results were expressed as mg of gallic acid equivalent per gram of the extract.[29]

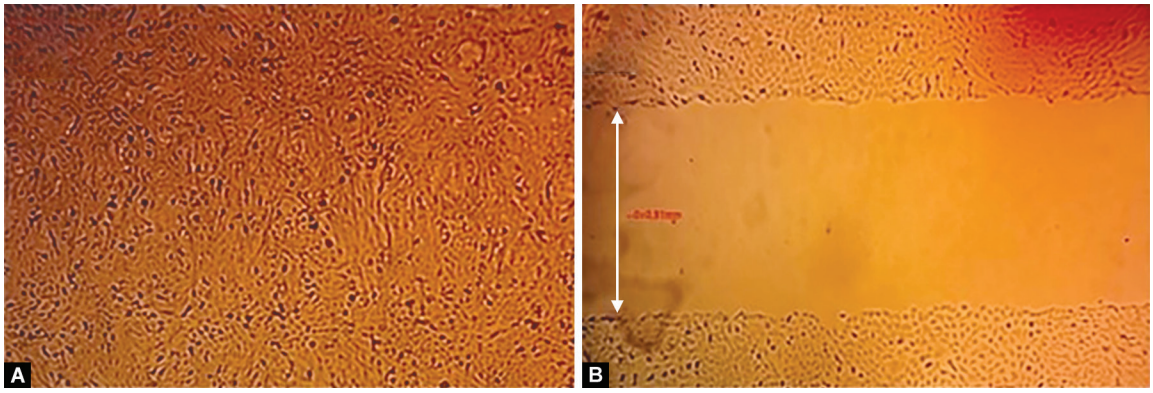
Determination of the Total Flavonoid Content

The total flavonoid content was determined using "aluminum chloride" colorimetric method. The GC extract was mixed with 1.5 mL of methanol, 0.1 mL of aluminum chloride (10%), 0.1 mL of 1-Molar solution potassium acetate and 2.8 mL of distilled water. The mixture was kept at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer (Jasco V-630 spectrophotometer, Japan). The total flavonoids content was calculated on the basis of the standard curve for quercetin and the results were expressed as mg of quercetin equivalents per gram of the seeds extract.[29]

Preparation of the Hydrogel Wound Dressing

A film-forming solution was prepared following the solvent casting technique described by Rhim.[30] Sodium alginate powder (Sisco Research Laboratories, India) was added to distilled water to form 2% (w/v) SA colloidal solution. Constant stirring was carried out until the powder dissolved completely. Glycerol (1% v/v) was added as a non-volatile plasticizer. This solution was used for the preparation of alginate dressing (control group). In order to prepare the GC-alginate dressing solutions (intervention groups), the prepared SA dressing solution was modified by addition of (25 µg/mL and 50 µg/mL) gardens cress seed ethanolic extract to prepare 25 GC and 50 GC dressings, respectively.

The prepared colloidal solutions were poured into glass Petri dishes and were dried in an incubator (CMB, Torre Picenardi, Italy) at 45°C for 48 hours until constant weight (20 gm) and separable films consistency was achieved. Specimens were cut from the films using a metallic ring (5 mm internal diameter and 2 mm height). The specimens were immersed in 5% (w/v) $CaCl_2$ solution for 5 minutes to allow for cross-linking of the alginate hydrogel. They were thoroughly washed with distilled water for 3 minutes to remove any remnants of $CaCl_2$ solution.[31,32] A schematic diagram of the steps of preparation of the hydrogel dressings specimens, SA, 25 GC, and 50 GC is illustrated in Figure 1.



Figs 2A and B: Scratch wound assay. (A) A confluent monolayer of human skin fibroblast cells; (B) The cell-free zone following the scratch introduced in the confluent monolayer (arrow)

Preparation of the COE-PAK Dressing (Positive Control Group)

Equal lengths of the base and catalyst pastes of COE-PAK, GC, USA were dispensed and mixed according to the manufacturer's instructions. Mixing was performed until a uniform homogeneous color was obtained. The mixed cement was placed using a stainless steel spatula into polyvinyl chloride (PVC) molds with 5 mm internal diameter and 2 mm thickness to prepare the COE-PAK dressing specimens.[27]

Surface Characterization of the Specimens

Scanning electron microscope (SEM) (Quanta 250 FEG Field Emission Gun, FEI, Netherlands) was used to examine the surface morphology of the prepared alginate specimens. This was coupled with energy dispersive X-ray analyses (EDX) unit for the elemental analysis of the surface of the specimens. The surface of the alginate dressings was sputter-coated with gold to make them electrically conductive. Accelerating voltage 30 kV, magnification of 500× and resolution for Gun.1n were used for the elemental analysis of the surface.[33]

Randomization

Simple randomization sampling was done using "random.org" website. This site allowed the investigator to randomly allocate samples of each group to be incorporated either in the cytotoxicity, *in vitro* wound healing, or the antibacterial tests. Accordingly, the samples were placed in sequentially numbered, opaque sealed envelopes for testing procedures.

Cell Viability Assay

Cell Culture

Human skin fibroblast (HSF) cell line was obtained and cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 100 mg/mL of streptomycin, 100 units/mL of penicillin and 10% of heat-inactivated fetal bovine serum. The cells were stored in a humidified incubator with 5% (v/v) CO_2 atmosphere at 37°C. The medium was routinely changed every 2 days and once confluence was reached; cells were subcultured (split ratio 1:2) by trypsinization (0.5% trypsin/0.02% EDTA). The third cell passage was used for the evaluation of cell viability and *in vitro* wound healing.[10]

Water-soluble Tetrazolium Salt (WST-1) Assay

Cell viability was assessed using WST-1 colorimetric assay at two different time intervals: 24 and 48 hours ($n = 15$). The surface of each specimen was sterilized by means of ultraviolet radiation (GIMA Lampada Germicidal lamp, wavelength (200–280 nm), Italy) for 30 minutes to avoid any contamination to the cells during the assay. Following sterilization, each specimen was added to a sterile tube containing 20 mL DMEM and stored for 24 or 48 hours to obtain specimen extracts in DMEM. An aliquot of 50 µL cell suspension was seeded in each well of the 96-well plates, then the plates were incubated for 24 hours. After 24 hours, either 50 µL DMEM (control) or specimen extracts in DMEM (tested) were added to the seeded cells. The cell culture plates were incubated at 37°C for another 48 hours. Subsequently, 10 µL of WST-1 reagent was added to each well of the 96-well plate and the absorbance value was measured after 1 hour at 450 nm using a fluorescent microplate reader spectrometer (Jasco V-630 spectrophotometer, Japan) to measure the optical density (OD). In order to calculate the cell viability percentage (%), the following equation was used.[10]

$$\text{Cell viability\%} = (\text{OD}_{\text{tested}} / \text{OD}_{\text{control}}) \times 100$$

In Vitro Wound Healing Assay (Scratch Wound Assay)

Human skin fibroblast cells were grown at a density of 3×10^5 cells/well onto 12-well plates and were cultured overnight. When confluence was reached, scratches were introduced mechanically into the formed confluent monolayers using a sterile pipette tip to introduce cell-free zones as shown in Figure 2. The plate was washed thoroughly with phosphate-buffered saline (PBS) to remove any cell debris. One milliliter of fresh media containing the dressing extracts was added to each well. Images (40× magnification) were captured using an inverted microscope (Labomed TCM400 Inverted Microscope, USA). The wound width, and migration rate were calculated at 0, 24, 48, and 72 hours' time intervals. Wound width was calculated as the average distance between the edges of the scratches using Fiji-ImageJ software.[34] Cell migration rate of the cells was calculated according to the equation described by Vidal et al.[35]

$$R_m = W_i - W_f / t$$

where R_m is the rate of cell migration, W_i is the initial wound width, W_f is the final wound width, and t is the duration of migration (in hours).

The plates were incubated at 37°C and 5% CO_2 in between the time intervals.[36]

Table 1: Compounds identified by analysis of garden cress seeds ethanolic extract by gas chromatography–mass spectrometry (GC/MS)

Rt (min.)*	*MW***	*MF****	*Area %*	*Identified compounds*
6.06	160	$C_7H_{12}O_4$	26.23	1,3-Propanediol diacetate
6.37	100	$C_6H_{12}O$	0.87	4-Methyl-1-penten-3-ol
6.67	142	$C_7H_{10}O_3$	49.62	Diallyl carbonate
10.63	175	$C_{10}H_9NO_2$	0.70	2-Phenyl-4-hydroxymethyl-1,3-oxazole
13.90	168	$C_{12}H_{24}$	0.43	1-Dodecene
14.11	170	$C_{12}H_{26}$	0.50	Dodecane
19.18	196	$C_{14}H_{28}$	1.16	4-Tetradecene (E)
19.37	172	$C_{11}H_{24}O$	0.78	Hexyl pentyl ether
22.39	206	$C_{13}H_{18}O_2$	1.47	3,4-Dihydro-2H-1,5-(3″-t-butyl) benzodioxepine
23.95	242	$C_{16}H_{34}O$	1.86	1-Hexadecanol
24.11	198	$C_{14}H_{30}$	0.55	Tetradecane
28.25	280	$C_{20}H_{40}$	3.92	5-Eicosene, (E)
28.38	212	$C_{15}H_{32}$	0.53	Pentadecane
32.24	284	$C_{18}H_{36}O_2$	4.28	Hexadecanoic acid, ethyl ester
35.27	294	$C_{19}H_{34}O_2$	0.60	9,12-Octadecadienoic acid, methyl ester (E,E)
35.40	306	$C_{20}H_{34}O_2$	4.15	Ethyl 9,12,15-octadecatrienoate
35.72	240	$C_{16}H_{32}O$	1.04	Hexadecen-1-ol, trans-9
35.81	312	$C_{20}H_{40}O_2$	0.67	Octadecanoic acid, ethyl ester
38.68	282	$C_{18}H_{34}O_2$	0.64	9-Octadecenoic acid (Z)

*R_t, retention time; **MW, molecular weight; ***MF, molecular formula

Antibacterial Test (Agar Diffusion Test)

Streptococcus mutans (ATCC 25175) and *Lactobacillus casei* (ATCC 9595) were used to evaluate the antibacterial action of the dressings. In the case of *S. mutans*, discs ($n = 15$) were placed onto the blood agar medium. The plates were incubated in the candle jar at 5–10% CO_2, 37°C and pH 5 for 24 hours.[37] On the other hand, the discs were placed onto the De Man, Rogosa and Sharpe (MRS) agar medium when using *L. casei* strain, and the plates were incubated for 24 hours at 7–15% CO_2, 37°C and pH 5 under anaerobic conditions using anaerobic gas generating sachets. For both strains, after the incubation period, the diameter of the inhibition zone in millimeters around each disc was measured.[37]

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 18. Numerical data were recorded as mean and standard deviation. Data were explored for normality by checking the data distribution and using Kolmogorov–Smirnov and Shapiro–Wilk tests. Comparisons between groups with respect to normally distributed numeric variables were compared by one-way analysis of variance (ANOVA) test, followed by Tukey's *post hoc* test. Comparison of different observation times was performed by repeated measures ANOVA test. p-values ≤ 0.05 were considered significant.

Results

Characterization of the Prepared Extract

Gas Chromatograph with Mass Spectrometer (GC/MS) Analysis

The compounds identified with the application of gas chromatography coupled with mass spectrometry (GC/MS) of the GC seeds ethanolic extract are listed in Table 1.

Total Phenols and Flavonoids Content

Results revealed that ethanolic extract of GC seeds contained 577 mg/100 gm total phenols, and 49 mg/100 gm total flavonoids.

Surface Characterization of the Prepared Dressings Using SEM with EDX

The SEM micrographs of the positive control group (COE-PAK) revealed a uniform surface (Fig. 3A). The EDX spectra and elemental analysis revealed the presence of the carbon (C) (67.62 at. %), zinc (Zn) (19.84 at. %), oxygen (O) (6.44 at. %), and magnesium (Mg) (4.86 at. %) (Fig. 3B).

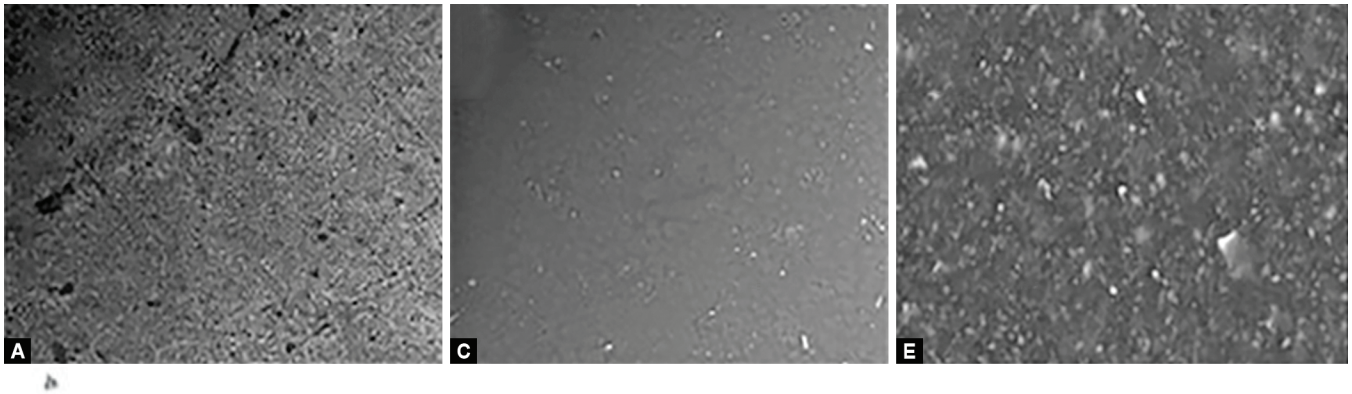
The scanning electron micrographs of the surface of the non-cross-linked alginate dressing before immersion in $CaCl_2$ solution revealed a uniform surface with almost homogeneous texture (Fig. 3C). After immersion in $CaCl_2$ solution, the surface was changed, but with a non-homogeneous texture (Fig. 3E). The EDX spectra and elemental analysis of the alginate dressings before immersion in $CaCl_2$ solution revealed the presence of carbon (C) (44.69 at. %), oxygen (O) (42.20 at. %), and sodium (Na) (13.11 at. %) (Fig. 3D). After immersion in $CaCl_2$ solution, calcium (Ca) (4.41 at. %) was detected. Na decreased to (8.48 at. %), while C and O were (41.89 at. %) and (45.22 at. %), respectively (Fig. 3F).

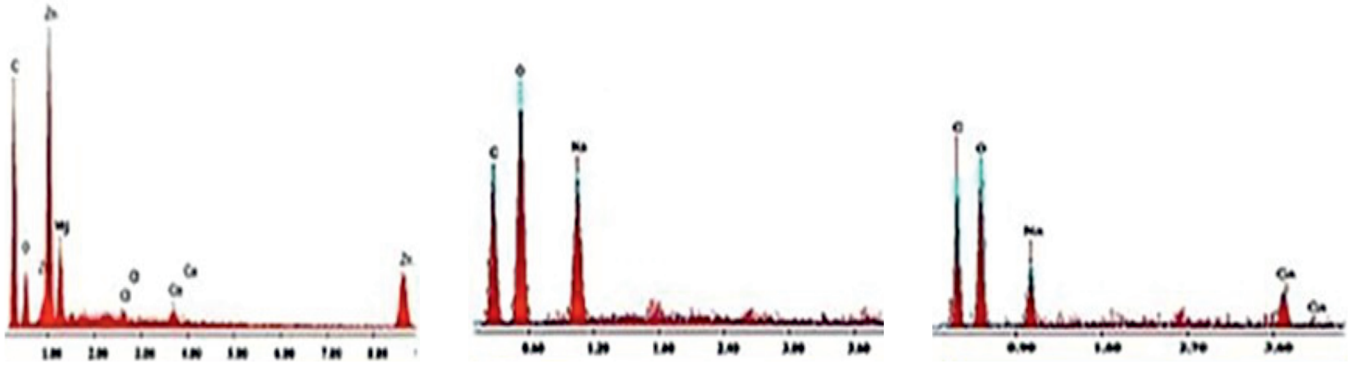
Cell Viability Assay (WST-1)

In terms of cell viability, no significant difference existed between all groups: COE-PAK, SA, 25 µg/mL garden cress (25 GC), and 50 µg/mL garden cress (50 GC) at both time intervals: 24 and 48 hours ($p = 0.24$ and 0.1, respectively). Results of the cell viability assay are listed in Table 2.

In Vitro Wound Healing Assay

The wound width decreased over time from 0 to 48 hours and was completely closed after 72 hours in all groups except SA group. The microscopic images revealing the change in the wound width





Element	Atomic %	Weight %
C K	67.62	34.16
Zn K	19.84	54.55
O K	6.44	4.34
Mg K	4.86	4.97
Total	100.00	100.00

B

Element	Atomic %	Weight %
C K	44.69	35.47
O K	42.20	44.61
Na K	13.11	19.92
Total	100.00	100.00

D

Element	Atomic %	Weight %
C K	41.89	31.48
O K	45.27	45.22
Na K	8.48	12.19
Ca K	4.41	11.06
Total	100.00	100.00

F

Figs 3A to F: SEM images (at 500×) and EDX spectra and elemental analysis of the dressings' surfaces. (A and B) COE-PAK dressing; (C and D) Alginate before immersion in $CaCl_2$ solution; (E and F) Alginate after immersion in $CaCl_2$ solution

Table 2: The mean, standard deviation (SD) and *p*-value of cell viability water-soluble tetrazolium salt (WST-1) of all groups ($n = 15$)

	Cell viability (%)								
	COE-PAK		*SA*		*25 GC*		*50 GC*		
Group	*Mean*[#]	*SD*	*Mean*[#]	*SD*	*Mean*[#]	*SD*	*Mean*[#]	*SD*	*p-value*
24 hours	99.50^{Aa}	0.36	99.02^{Aa}	0.70	98.58^{Aa}	0.78	98.89^{Aa}	2.20	0.24 ns
48 hours	98.73^{Ba}	0.54	98.49^{Ba}	0.51	98.81^{Aa}	0.85	98.58^{Aa}	0.38	0.1 ns
p-value	0.014*		0.005*		0.341 ns		0.568 ns		

Superscripts with different capital letters indicate statistically significant difference within the same column. Superscripts with different small letters indicate statistically significant difference within the same row. Significance level $p \leq 0.05$, *significant, ns, nonsignificant. [#]($n = 15$)

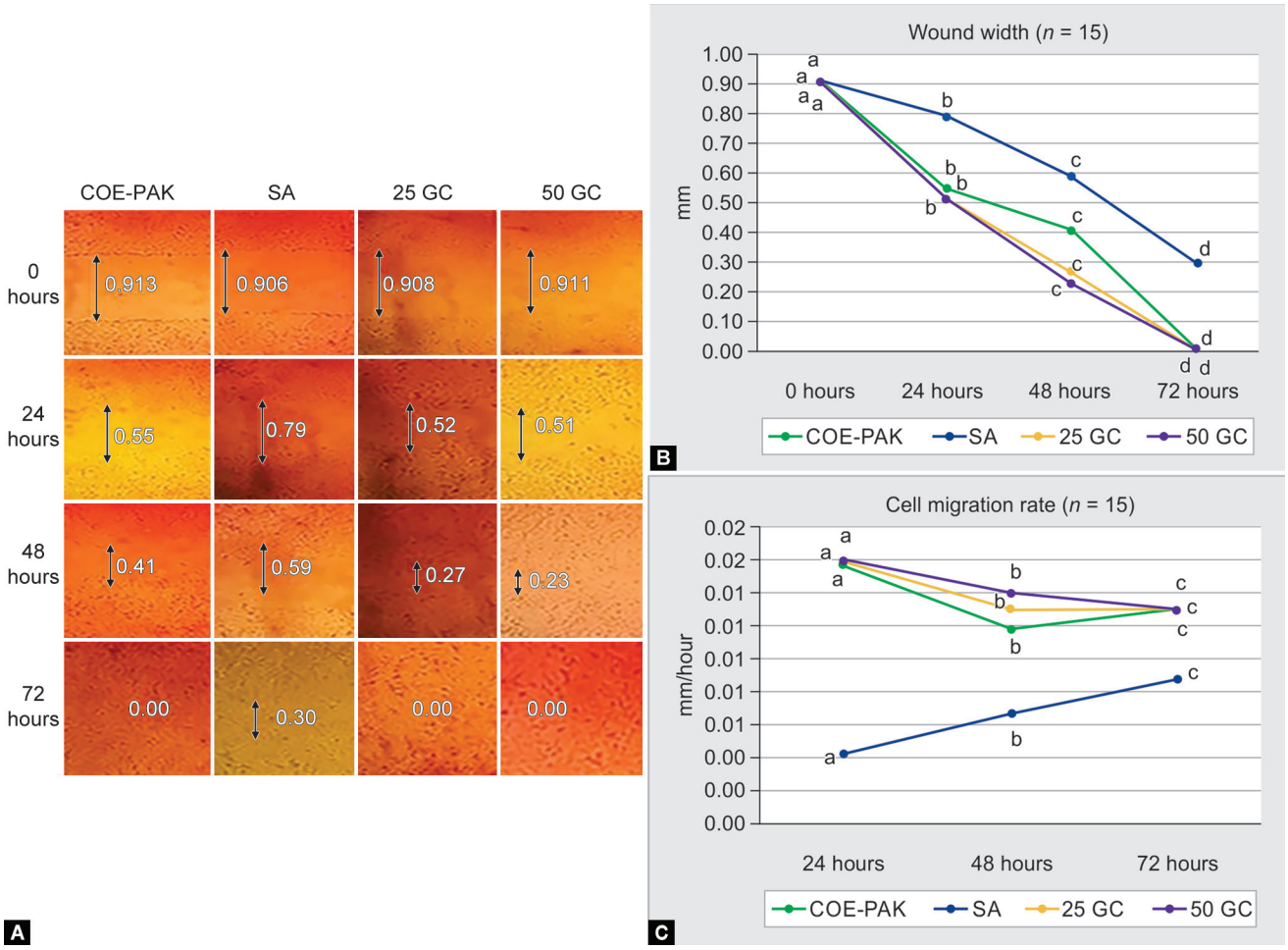
are shown in Figure 4A. Results of the wound healing assay are shown in Figures 4B and C. Results revealed a statistically significant difference in the wound width and cell migration rate mean values between all groups ($p \leq 0.05$). The GC-containing groups recorded the lowest mean values in wound width (0.27 mm ± 0.01 for 25 GC and 0.23 mm ± 0.01 for 50 GC at 48 hours) and the highest mean value of cell migration rate (0.013 mm/hour).

Antibacterial Action (Agar Diffusion Test)

Inhibition zones (mm) were detected around the specimens in both the *Streptococcus mutans* and *Lactobacillus casei* Petri dishes. The smallest inhibition zones were detected around COE-PAK, followed by SA and 25 GC groups, with the largest ones were detected around 50 GC specimens in both strains. Results of the antibacterial action are shown in Figure 5.

Discussion

Although COE-PAK is considered the gold standard of oral dressing materials, it has moderate to severe cytotoxicity[27,38] with questionable antibacterial action.[39] Natural products are one of the most effective alternatives to replace synthetic cytotoxic agents. Although alginate has several medical applications,[10,40] it



Figs 4A to C: Results of the wound healing test (scratch wound assay). (A) Microscopic images (40x) of well plates revealing the wound width (mm) of the different groups: COE-PAK, sodium alginate (SA), 25 µg/mL garden cress (25 GC) and 50 µg/mL garden cress (50 GC). The black arrows denote the cell-free zone; (B) Line chart revealing the mean wound width in different groups at different time intervals; (C) Line chart revealing the mean cell migration rate in different groups at different time intervals

For each line, within each group, different letters denote significant difference at the different time intervals

lacks the potent antibacterial action and wound healing potential. On the other hand, GC is rich in minerals, phenolic and flavonoid compounds, and essential elements necessary to promote healing and tissue repair together with antibacterial action.[22,41,42] Therefore, the aim of the current study was to evaluate the use of GC ethanolic extract incorporated in SA as a wound dressing compared with COE-PAK.

In the current study, ethanol was used for GC seeds extraction. Ethanol is more effective in extraction of the fatty acid profile of the plants than water and less cytotoxic than methanol.[43]

The prepared extract was characterized using gas chromatography with mass spectrometry (GC-MS), which is a valid analytical method for the analysis of multicomponent mixtures and volatile organic compounds.[44] The obtained results (Table 1) were similar to those reported by Al-asmari et al. Abu-Rumman and Abdallah et al. thus confirming the composition of GC seeds.[18,45,46]

Alginate was used as a vehicle for the GC extract, because alginate is biocompatible, with the ability to release drugs in a controlled manner.[47] During preparation, glycerol was added to the SA colloidal solution, to act as a plasticizer. This may have contributed to the preparation of a flexible polymeric material, making it more suitable for manipulation as a wound dressing.[48] Glycerol was selected since cytotoxicity results revealed no toxicity of glycerol to HSF.[49] The films were prepared by means of the "solvent casting" method to allow for physical evaporation of the water from the colloidal solution. As water evaporated from the colloid, there was an observed increase in the viscosity due to a higher concentration of polymer and a thick semisolid mass was a transition stage until constant weight was obtained to reach the film stage.

Immersion of the films in calcium chloride solution was employed for cross-linking of SA in order to convert the soluble SA into insoluble calcium alginate. Successful cross-linking was confirmed by the EDX results, where the calcium content increased (4.41 at. %) following immersion in $CaCl_2$ solution. After immersion, washing was done with distilled water, as the calcium chloride solution is cytotoxic, and hence may interfere with the cytotoxicity results.[50,51] Absence of Cl element as revealed by EDX (Fig. 3F) may confirm complete elimination of calcium chloride solution from the surface of the dressings.

The SEM images (Fig. 3) revealed uniform surfaces of the dressings, which fulfills the requirement of wound dressing as

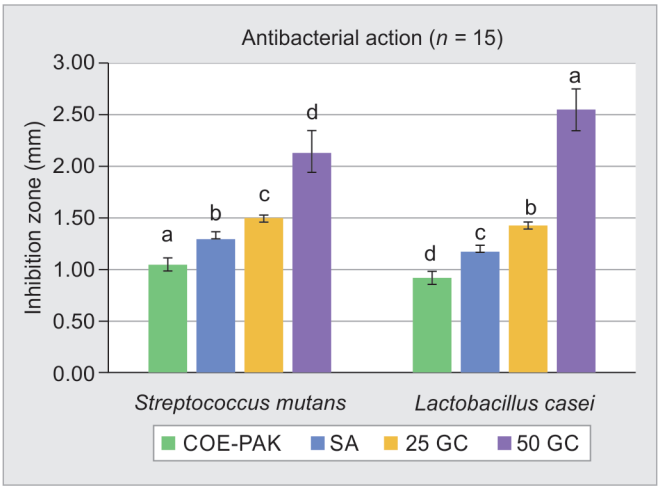


Fig. 5: Bar chart illustrating the mean value of antibacterial action in different groups: COE-PAK, sodium alginate (SA), 25 µg/mL garden cress (25 GC), and 50 µg/mL garden cress (50 GC) against *Streptococcus mutans* and *Lactobacillus casei*

Different letters denote significant difference: within the same strain between materials and within the same material between strains

being non-retentive to bacteria, and hence may prevent plaque accumulation. However, the surface texture of the alginate became non-homogeneous after immersion in $CaCl_2$ solution. This may be due to the formation of a network of alginate chains.[33]

In the current study, fibroblasts were selected to assess the cell viability of the dressings since they play a vital role in the initial stages of wound healing.[52] Cell viability was evaluated using WST-1 rather than MTT assay, since WST provides more accurate results with fewer steps. Results revealed no significant difference in the cell viability between the all groups. Cytotoxicity results failed to reject the null hypothesis. In the current study, the cell viability test of COE-PAK using HSF and WST-1 assay revealed 99.5% ± 0.36 at 24 hours and 98.99%s ± 0.54 at 48 hours. These results are higher than those reported in the literature by Kadkhodazadeh et al. and Alpar et al. using MTT assay.[27,53] This difference may be due to the use of different assays and/or different cell types, or may be due to the fact that MTT reagent is more cytotoxic.[54] The statistically significant higher cell viability value of COE-PAK in 24 hours (99.50% ± 0.36) compared with 48 hours (98.99% ± 0.54) may be due to the release of more rosin present in COE-PAK as it was reported to be a cytotoxic agent.[55] The same pattern observed in SA may be due to the release of some of the calcium ions used for cross-linking, as it was reported that calcium ions could inhibit the proliferation of cells in alginate hydrogel by osmosis effect.[56] On the other hand, cytotoxicity of the GC groups (25 GC and 50 GC) was the same after both 24 and 48 hours. Such findings may confirm that these percentages were within the biologically safe concentration.

Although the wound healing assay is widely applied, it lacks standardization in the procedure approach, making it difficult to achieve meaningful comparisons among results in reported literature.[36] The cell-free zone in the current study was created mechanically rather than by physical exclusion method, since the mechanical technique is more clinically relevant resembling the surgical procedure and provides well-defined gap edges.[36] However, since the scratch is created manually, care was taken to generate reproducible wounds as was confirmed by the nonsignificant difference in the wound width at 0 hour (Fig. 4B).

Washing with PBS was done to obtain a clean wound free from cellular debris. This would ensure reliable results.[57] The *in vitro* wound healing was microscopically evaluated using an inverted microscope as described by Harishkumar et al. and Bolla et al. [52,58]

Results of the wound healing assay revealed a significant reduction in the wound width with higher cell migration rate values in the GC-containing groups compared with the COE-PAK and SA groups (Figs 4B and C). This may be attributed to the presence of oleic and linolenic acids in the prepared GC seeds ethanolic extract, as revealed by GC/MS analysis [9•Octadecenoic acid (Z)], and (Ethyl 9,12,15octadecatrienoate), respectively (Table 1). Researches revealed that oleic acid could increase cell proliferation and migration by increasing the formation of focal contacts.[59,60] Dixit et al. and Juma reported that GC induced faster healing of bony wound compared with the control group.[41,61] They attributed their findings to the oleic and linolenic acids content of GC. This may also explain the smaller wound width and higher cell migration rate results obtained by the 50 GC group compared with 25 GC group, as 50 GC contains higher content of oleic and linolenic acids compared with 25 GC. The delayed healing with greatest wound width and least fibroblast migration rate, obtained by SA group, may be attributed to the lack of any active constituents that promote healing and cell proliferation. This finding was in accordance with the results reported by Zhang et al., who revealed that SA had no effect of fibroblast proliferation.[62] Although our results revealed a significant difference in wound healing in terms of wound width and cell migration rate, clinical significance has not been investigated yet. However, it is believed that GC will also have better clinical performance regarding healing. Further clinical studies need to be conducted to prove such hypothesis.

Streptococcus mutans and *Lactobacillus casei* are the main microorganism involved in infected oral wound.[63] Hence, they were the selected strain for the antibacterial test in the current study. COE-PAK showed the least antibacterial action against both strains. This may confirm the questionable bacteriostatic action of COE-PAK despite the addition of chlorothymol as claimed by the manufacture. The SA group possessed lower antibacterial action against both strains. This may be due to the fact that alginate is a negatively charged material that affects bacterial cell membrane permeability.[64] The GC-containing dressings possessed the highest antibacterial activity against both strains. This may be attributed to their ability to inactivate cell membrane transport proteins and enzymes by the action of phenolic and flavonoids compounds[65,66] as revealed from chemical analysis. These findings were in accordance with those reported by Besufekad and Akrayi et al. who reported effectiveness of GC against both gram-positive and gram-negative bacteria.[66,67] In addition, phytochemical analysis of GC seeds by GC/MS revealed the presence of alkaloids (2.Phenyl.4.hydroxymethyl-1,3-oxazole) and fatty acid methyl esters (9,12-octadecadienoic acid, methyl ester) (Table 1). These compounds are biologically active with a potent antibacterial action,[68] as they disturb both the electron transport chain and the oxidative phosphorylation processes, which are essential for energy production in bacterial cells. The fatty acid methyl esters bind to electron carriers or alter membrane integrity by decreasing the membrane potential and proton gradient.[68,69] Results of the

antibacterial action of the GC-containing dressings revealed that it could be helpful after periodontal surgery and in the case of oral biopsy. Studies revealed that the anticancer effect of GC and most of cancer patients are immunocompromised and are susceptible to bacterial infection. However, further clinical studies are required to evaluate its clinical performance and confirm such recommendations.

The limitation of the current study is that the natural materials as alginate and GC may have an intrinsic variability in composition and properties owing to geographical location and season. In addition, the complex intraoral environments cannot be fully replicated as this was an *in vitro* study. Therefore, *in vivo* testing is required to evaluate the tissue reaction to the prepared dressing after surgical operations.

Conclusion

The *in vitro* procedure used in the current study appeared to be suitable for preparation of a hydrogel wound dressing. Garden cress incorporated in SA (25 µg/mL and 50 µg/mL) may be a promising natural alternative to COE-PAK as wound dressing in terms of wound healing and antibacterial action against *S. mutans* and *L. casei*.

Clinical Significance

In this *in vitro* study, a natural wound dressing was prepared from SA modified with GC seeds extract, which can be clinically supplied as cross-linked, ready-to-use films that does not require mixing, eliminates human variations and save time. The biocompatibility and improved wound healing potential together with the antibacterial action of such a dressing, could make it the dressing of choice following periodontal surgery, with potential future prospective application in the field of oral cancer following oral biopsy. Furthermore, the use of natural alternatives is more environmentally friendly approach with less side effects.

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