

Original Research Article

Therapeutic potential of oleic acid nanovesicles prepared from petroleum ether extract of *Sargassum binderi* in streptozotocin-induced diabetic wound in Wistar rats

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Abstract

Purpose: To study the effectiveness of phyto-oleic acid nanovesicles (PONVs) developed from *Sargassum binderi* (an alga) in healing diabetic wound in a rat model, and to establish the associated changes in cytokine network.

Methods: Phyto-extract was obtained from the whole plant of *Sargassum binderi* by Soxhlet extraction using petroleum ether as solvent. The crude extract was subjected to phytochemical analysis and used in the formulation of PONVs. The PONVs were formulated by entrapping petroleum ether extract of *Sargassum binderi* using the film hydration technique. Wound healing property was determined by measuring both pro-inflammatory and anti-inflammatory cytokines using enzyme-linked immunosorbent assay (ELISA).

Results: Tannins and steroids were the major components of the petroleum ether extract of *Sargassum binderi*. Serum cytokine levels were increased after inducing diabetes and creating the wound. The serum levels of IL-2, TNF- α and IL-1 β were 37.3 ± 3.3 , 76.3 ± 5.2 and 3307.6 ± 350 pg/ml, respectively. Treatment with PONVs modulated the serum cytokine levels through significant decreases in serum IL-2, TNF- α , IL-1 β levels, and significant elevation of serum IL-4.

Conclusion: These results indicate that PONVs have promising potentials for application as topical treatment for diabetic wounds.

Keywords: Brown algae, *Sargassum binderi*, Oleic acid nanovesicles, Diabetic wound, Cytokines

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INTRODUCTION

Wound healing is a series of complex processes characterized by various phases such as

inflammation, epithelialization, angiogenesis, matrix deposition, and remodelling [1]. Wounds in diabetes mellitus have significant complications that are highly linked to

neuropathy [2]. Progression in the healing of diabetic wounds is associated with decreased keratinocytes, fibroblast migration of immune cells into the endothelial cells, and angiogenesis [3-5]. Cytokines are small molecular weight proteins that can reach the wound cells through autocrine, paracrine, and endocrine fashions, which are crucial factors in the inflammatory response. Specifically, cytokines have prominent roles in the wound healing process, ranging from pro-inflammatory to anti-inflammatory responses. The present state of treatment for diabetic wounds still faces significant challenges such as poor entry of drugs into the wound cells, and development of drug resistance.

Research efforts have resulted in the development of newer and wider-spectrum antibiotics. However, there is no assurance that wound-associated organisms will not develop resistance to these newer antibiotics [6]. Moreover, although the use of drugs of herbal origins as alternatives for the development of antibacterial agents has almost reached its maximum potential, their effectiveness is debatable till date. Consequently, researchers on drug discovery have shifted their attention to seaweeds for the past three decades, with the aim of developing antibacterial agents to replace the present antibiotic treatment regimen. In this respect, biomolecules with antibacterial properties and modulating effects on cytokines are highly significant in therapeutic management options for diabetic wounds.

The potential of *Sargassum binderi* which is available in the Red Sea of Jazan, Saudi Arabia, has not yet been explored as a pharmaceutical agent. The antibacterial effects of petroleum ether extract and oleic acid vesicles of *Sargassum binderi* have been demonstrated by Sivakumar *et al* [7]. In the present study, the immuno-regulatory effect of oleic acid vesicles prepared by entrapping petroleum ether extract of *Sargassum binderi* was investigated. In addition, the wound-healing effect of the oleic acid vesicles was studied in streptozotocin (STZ)-induced diabetic wounds in Wistar rats to ascertain their potential as topical application for treating diabetic wounds.

EXPERIMENTAL

Collection and identification of seaweed

Sargassum binderi was collected from the Red Sea, Al Murjan beach, Jazan, Saudi Arabia, and washed thoroughly using fresh water to remove extraneous substances and epiphyte adherents on the samples. *Sargassum binderi* Sonder ex J.

Agardh was identified by Dr. Remesh Moochikkal, herbarium curator, Herbarium of Jazan University, Department of Biology, Jazan University, Jazan, Saudi Arabia. A voucher specimen with ref no. 1210(JAZUH) was deposited at the institution's herbarium for future reference.

Phytochemical extraction

The seaweed was subjected to air-drying under shade for 15 days, after which the sample was sliced into small pieces and powdered using a grinder. The coarsely-powdered samples were pooled and kept in an airtight container. The powdered sample was extracted in a Soxhlet apparatus using hot, continuous percolation with petroleum ether. In essence, 200 g of dried *Sargassum binderi* powder was placed in a Soxhlet apparatus and extracted with petroleum ether as a solvent. The total ensemble was set up on a heating mantle. The solvent was heated to about 60 °C, and the solvent extraction was performed continuously for 4 h. Several 200-g batches of powder were similarly extracted. After extraction, the extracts were transferred into separate glass beakers which were kept open to permit solvent evaporation and air-drying of the extract. The dry samples were collected by scraping them off from the glass beaker, and were pooled and weighed. The pooled sample was then subjected to phytochemical analysis, and its PONV's formulations for diabetic wound healing.

Preparation of oleic acid vesicles

The PONV was prepared by using the film hydration technique. The extract and oleic acid were mixed in a 1:1 proportion. The mixture was sonicated for about 5 min and then mixed with methanol in 1 : 1 proportion. The mixture was heated on a heating mantle at 50 °C for 60 min to form a thin film following solvent removal. Finally, the PONV was eluted in methanol and the resultant vesicles were used for *in vivo* studies on wound healing properties.

In vivo wound healing property

Healthy male Wistar rats weighing 170 – 200 g were used. They were acclimatized to laboratory conditions and handled in strict compliance with the guidelines of the International standard and Institutional Animal Care and Use committee (IACUC) [8]. The rats were assigned randomly to three different groups (6 rats/group) and categorized as follows:

Group 1: Normal control group: The animals did not receive streptozotocin (STZ) or PONVs.

Group 2: Disease control group (diabetes induction and diabetic wound): The rats received intraperitoneal injection of STZ (at a dose of 50 mg/kg body weight dissolved in 0.9 % w/v of sodium chloride (normal saline). The wound was created according to the procedure established by Mendes *et al* [9]. In this process, the rats were anesthetized with intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (25 mg/kg). Then, the dorsal surface hair was trimmed with an electric shaver, followed by hair removal using commercially available hair remover. An area of skin was cleaned with 10 % povidone iodine solution, and a 6-mm diameter skin was removed with a sterile surgical blade to create a circular wound on the upper back of the rat (Figures 2 A & B).

Group 3: Treatment group: The rats received intraperitoneal injection of STZ (50 mg/kg body weight) dissolved in normal saline, and diabetic wound as in group 2. In addition, they were treated with 500 μ l of PONVs (twice daily) by spreading them on the wound for seven days. After induction of diabetes, blood glucose levels were measured at predetermined time intervals in the morning and evening. The level of glucose was measured on the tail-vein blood using a standard commercially available glucometer.

The blood glucose level was checked and the diabetes was confirmed by the elevation of fasting blood glucose above 250 mg/dL. On day 6, fasting blood samples were collected and allowed to clot. The serum samples obtained by centrifugation were kept in a refrigerator at 2 – 8 °C prior for use in the assay of cytokine levels. The pro-inflammatory cytokines, IL-1 β , IL-2, IL-4, and TNF- α levels were estimated as follows.

Serum IL-1 β

The level of IL-1 β in serum was measured quantitatively by using IL-1 β rat ELISA kits (ABCAM, USA). The assay employs simple step sandwich ELISA to determine the IL-1 β level in the serum sample. Simultaneously, standards and samples were pipetted into the respective wells and incubated at room temperature for 2.5 h after closing the ELISA plate with a lid. During incubation, the IL-1 β present in the sample was bound to the wells that had been coated with the immobilized specific antibody. After incubation, the wells were washed thoroughly with 1X washing solution using Biotek ELISA washer (Elx50, USA). Then, 1X biotinylated anti-rat IL-1 beta antibody was added to each well. The plate

was incubated for 1 h at room temperature with mild shaking.

After incubation, the plate was washed as previously described, and the unbound biotinylated antibody was removed. Then, horseradish peroxidase (HRP)-conjugated streptavidin was added to the wells, and the wells were again washed as described in the previous step. A substrate solution, tetramethylbenzidine (TMB) was added to the wells and incubated for 30 min at room temperature in a dark with mild shaking. This was followed by addition of the stopping solution. The thickness of the developed colour which is proportional to the amount of IL-1, was measured at 450 nm using a Biotek ELISA reader (ELX 800, USA). The absorbance was an indication of the amount of IL-1 beta bound to the specific antibody. The sample concentration was calculated by extrapolation from a standard curve.

Serum IL-2

The level of IL-2 in serum was estimated quantitatively using IL-2 rat ELISA kit, ABCAM, USA. Sandwich ELISA was used to determine the IL-2 level in serum samples. Standards and samples were pipetted simultaneously and dispensed into the respective wells. The plate was closed with a lid and incubated at 4 °C overnight with mild shaking. Thereafter, the solution was discarded and the wells were washed with 1X washing solution using Biotek ELISA washer ELX 50, USA. Then, 1X biotinylated anti-rat IL-2 antibody was added and incubated at room temperature for 1 h with mild shaking. The plate was washed as previously described, and the unbound biotinylated antibody was removed. Then, HRP-conjugated streptavidin was added to the wells, followed by incubation for 45 min at room temperature.

The wells were again washed as described in the previous step. Finally, TMB solution was added to all the wells, followed by incubation in the dark with mild shaking at room temperature for 30 min. Thereafter, the stopping solution was added and the concentration of IL-2 was measured by reading absorbance at 450 nm using a ELISA reader (Biotek ELX 800). The concentration of IL-2 was obtained by extrapolation from a standard curve.

Serum IL-4

Serum IL-4 was quantitatively estimated in a sandwich ELISA assay using IL-4 rat ELISA kits (ABCAM, USA). The procedure was similar to that used for IL -2 estimation, except that 1X

biotinylated anti-rat IL-4 antibody was added. Absorbance of the colour developed was read at 450 nm using a ELISA reader (Biotek ELX 800). The concentration of TNF- α was obtained by extrapolation from a standard curve.

Serum TNF- α

Serum TNF- α level was quantitatively estimated using a simple step sandwich ELISA assay. Samples and standard were simultaneously pipetted into the respective wells. Then, 1X biotinylated anti-TNF- α was put in the wells and the plate was incubated for 3 h at room temperature. Thereafter, the plate was rinsed thrice with 1X washing Buffer using Biotek ELISA washer (ELX50, USA), followed by addition of 1X streptavidin-HRP solution to the wells. The plate was closed and incubated at room temperature for 30 min, after which it was rinsed with washing buffer as described earlier. Then, TMB substrate solution was added to all the wells, followed by incubation in the dark at room temperature for 20 min. The reaction was stopped by addition of the stopping solution, and the absorbance of the resultant color was read at 450 nm using an ELISA reader (Biotek ELX 800). The concentration of TNF- α was extrapolated from a standard curve.

Statistical analysis

The data are presented as mean \pm standard deviation (SD). The statistical significance of differences between treatment groups were determined using one-way analysis of variance (ANOVA) and Dunnett multiple comparison test. All analyses were performed with GraphPad InStat 3.10 software, USA. The level of significance was set at $p < 0.001$.

RESULTS

Results from hot continuous percolation method showed that the petroleum ether extract of *Sargassum binderi* predominantly contained

tannins and steroids. The PONVs were successfully formulated. The *in vivo* wound healing study revealed that PONVs possess significant wound-healing properties. The results shown in Table 1 indicate that group 2 rats treated with STZ exhibited very high and significant increases in the serum levels of IL-1 β , IL-2, and TNF- α ($p < 0.001$).

However, in the treatment group (group 3), exposure to PONVs successfully reversed these increases by significant decreases in serum levels of IL-1 β , IL-2, and TNF- α . The decreases in these pro-inflammatory cytokines were consistent with the wound-healing results of PONVs as depicted in Table 1. Interestingly, PONVs treatment led to significant increases in the serum level of the anti-inflammatory cytokine IL-4 ($p < 0.001$), when compared with the other groups (Table 1). The results in Figure 1 demonstrate stepwise, the *in vivo* wound-healing process of PONV as self-exemplary.

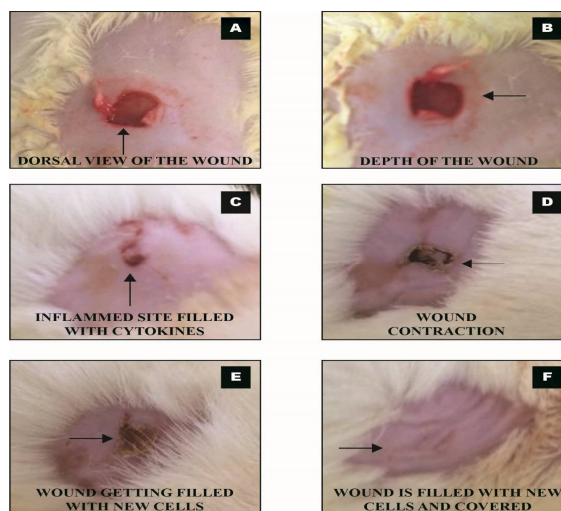


Figure 1: Wound healing property of PONV in various stages of healing processes of diabetic wound. Fig1 (A&B) represents the diabetic wound created in rats; Fig (C to F) represents the wound healing processes

Table 1: Pro-inflammatory and anti-inflammatory cytokine levels before and after treatment of PONV in various treatment group

Cytokine level (pg/ml)	Treatment groups			P-value
	Group 1	Group 2	Group 3	
IL-2	1.48 \pm 0.5	37.3 \pm 3.3	1.688 \pm 0.4**	<0.001
TNF- α	2.49 \pm 0.61	76.3 \pm 5.2	3.25 \pm 0.65**	<0.001
IL-1 β	106.3 \pm 6.8	3307.6 \pm 350	137.7 \pm 23.3**	<0.001
IL-4	1.67 \pm 0.28	27 \pm 3.63	38.35 \pm 5.34**	<0.001

Each value is e mean \pm SD ($n = 6$); $p < 0.001$ (99.9% confidence interval) is very extremely significant when compared to normal group. *Fold decreased in concentration (pg/ml) after treatment # Fold increased in concentration (pg/ml) after treatment

DISCUSSION

The present study focused on establishing the effect of PONVs on wound healing in diabetic rats by modulating pro-inflammatory cytokine levels. Streptozotocin (STZ) is a potent chemical popularly used for the induction of diabetes mellitus. It produces a toxic effect by destructing the β cells of the islet of Langerhans [10,11]. In this study, STZ-treated rats significantly developed diabetes mellitus. It is known that IL-1 β is a predominant cytokine present in inflammatory conditions linked to diabetic mellitus [12].

Pro-IL-1 β is an inactive precursor protein that is converted by the enzyme caspase 1 to active IL-1 β which is associated with defective secretion of insulin as well the development of insulin resistance [13–15]. In the present study, serum IL-1 β level was upregulated expressively in the STZ-injected rats, when compared to the normal control group. However, treatment with PONV brought about significant reduction in serum IL-1 β level. Generally, T Helper 2 (CD4+) cells predominantly produce a low molecular weight protein called interleukin-2 (IL-2). Cytotoxic T (CD8+), natural killer (Nk), and natural killer T (Nk T) cells are also involved to a lesser extent in the production of IL-2 which has pro-inflammatory and anti-inflammatory effector functions.

It has been shown that IL-2 is a messenger that provides signals to various lymphocytes during proliferation and differentiation, leading to immune responses and maintenance of homeostasis [11]. The present study showed that the IL-2 level increased in the group 2 animals that received STZ but the increase was reversed significantly after treatment with PONV in group 3 animals. It has been suggested that IL-2 signals affect the action of CD8+ cells while mounting the immune response and therefore, the inflammatory response action is ineffective [16]. In contrast, the present study showed the predominant inflammatory effect of IL-2 which was significantly reduced by treatment with PONV.

The cytokine TNF- α , a low molecular weight protein derived mainly from macrophages, is involved in inflammatory mechanism and closely associated with insulin resistance [17]. It is an inflammatory biomarker that plays a role in regulating the inflammatory processes in diabetes [18]. Consistent with the earlier report, the present study showed that STZ-induced diabetes was accompanied with significant increases in serum TNF- α level. The results

indicate that the STZ treatment induces insulin resistance. It is very interesting to note that after treatment with PONVs, the reduction in serum TNF- α level and the attendant reduction in inflammation occurred with healing of the diabetic wound. Interleukin-4 (IL-4) plays an important role in regulating the anti-inflammatory responses and growth factor for both B and T lymphocytes.

It has been reported that the production of IL-4 is very high in diabetes mellitus accompanied with chronic periodontal disease [19]. Interestingly, the present study has demonstrated that IL-4 level was very high after the treatment with PONVs, when compared to the untreated STZ-induced diabetic group. Thus, PONVs are potent immune boosters which modulate the inflammatory response and heal wounds. In a previous study, it was reported that IL-4 inhibited the secretion of IL-1 β and TNF- α using activated monocytes [20]. Similarly, the present study showed that after treatment with PONVs, serum IL-4 level increased while serum IL-1 β , IL-2, and TNF- α levels decreased. It has been reported that IL-2 plays an important role in Th2 cell differentiation that stabilizes the accessibility of the IL-4 gene and efficiently primes IL-4 production [21,22].

CONCLUSION

The results obtained in the present study indicate that the phyto-extract of *Sargassum binderi* entrapped in oleic acid nanovesicles (PONVs) possesses effective *in vivo* wound-healing property in a diabetic rat wound model. The wound-healing occurred via a mechanism involving downregulation of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities

pertaining to claims relating to the content of this article will be borne by them.

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