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Effect of Vitamin D on the Immunomodulatory and Antibacterial Activities of Peripheral Blood Mononuclear Cell Secretions Against Multidrug-Resistant Acinetobacter baumannii Isolated from Chronic Wound and Burn Infections

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Abstract

Acinetobacter baumannii is a multidrug-resistant bacterium commonly linked to chronic wound and burn infections, recognized for its capacity to produce biofilms that impede wound healing. Vitamin D possesses immunomodulatory characteristics that may augment host defensive mechanisms. This in vitro study examined the influence of vitamin D on the secretions of peripheral blood mononuclear cells (PBMC) and their immunological and antibacterial efficacy against Acinetobacter baumannii. Peripheral blood mononuclear cells (PBMCs) were extracted from ٥ donors and cultivated under controlled laboratory conditions. A total of 100 chronic wound samples were collected, resulting in the identification of 40 A. baumannii isolates. Cytokine concentrations (IL-6, IL-1β, and IFN-γ) were quantified via ELISA prior to and after to vitamin D intervention. The antibacterial and antibiofilm properties of PBMC secretions were assessed. Vitamin D therapy decreased IL-6 levels from 585 to 410 pg/mL (29.9%), IL-1β levels from 185 to 135 pg/mL (27.0%), and IFN-γ levels from 440 to 280 pg/mL (36.4%). PBMC secretions suppressed the proliferation of A. baumannii and diminished biofilm development. Secretions generated from monocytes demonstrated increased antibacterial efficacy after vitamin D administration. Vitamin D regulates PBMC function by diminishing pro-inflammatory cytokine synthesis and augmenting antibacterial efficacy against A. baumannii. The findings indicate that vitamin D-treated PBMC secretions may serve as a promising supplementary strategy for addressing persistent wound infections caused by multidrug-resistant bacteria.

Keywords: *Acinetobacter baumannii,* ELISA, immune cells , secretions, vitamin D, wound infection.

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Introduction

Human skin serves as an effective barrier against infection, protecting the underlying tissues, bones, and organs [1]. A wound is a break in the skin's or tissues' structural integrity that reduces the skin's ability to protect itself [2]. After burn injuries, wound infections account for 70% to 80% of surgical patient deaths and morbidity [3]. Over the past thirty years, Acinetobacter baumannii has gained recognition as an increasingly important opportunistic human pathogen[4].

Worldwide, an increasing number and severity of A. baumannii outbreaks have occurred in various settings, such as hospitals and long-term care facilities [5]. A. baumannii's ability to persist in harsh environmental conditions coupled with its alarmingly rapid rate of antibiotic resistance acquisition places this pathogen as one of the six most problematic multi-drug resistant (MDR) healthcare-associated pathogens worldwide [6]. Recent data from Iraq identify highly resistant strains of A. baumannii to be some of the most common organisms causing severe and often lethal wound infections[٧]. The virulence factors of A. baumannii responsible for the long-term survival and wide-spread transmissibility in the health care environment include the A. baumannii's ability to attach to and persist on solid and otherwise abiotic surfaces in the form of a biofilm as well as its ability to resist desiccation and disinfection [٨]. A. baumannii has been shown to easily spread from infected patients to the environment through the aerosol route and can survive in the nosocomial environment for up to 13 days[٩]. Chronic wounds are those that do not advance through the typical stages of healing within a designated timeframe and are sometimes compounded by ongoing bacterial infections and biofilm development[١٠] . Antimicrobial resistance (AMR) is one of the most serious risks to worldwide public health in the twenty-first century [١١]. Consequently, alternative therapeutic strategies that augment host immune responses are being actively explored to address multidrug-resistant infections.

Vitamin D is widely acknowledged as a significant immunomodulatory chemical that governs both innate and adaptive immune responses[12]. Its biological activity is facilitated by binding to the vitamin D receptor (VDR), which is present on several immune cells, including monocytes, macrophages, and lymphocytes[13]. The activation of VDR affects cytokine production, mitigates excessive inflammatory responses, and strengthens antimicrobial defense mechanisms[14]. Moreover, vitamin D has been demonstrated to stimulate the expression of antimicrobial peptides, including cathelicidin (LL-37), which facilitate bacterial clearance and wound protection[15]. Peripheral blood mononuclear cells (PBMCs) release a diverse array of bioactive molecules, encompassing cytokines, growth factors, and antimicrobial agents that are integral to immune regulation and tissue repair[16]. Recent research indicate that secretions generated from PBMCs may have therapeutic promise in wound healing, tissue regeneration, and infection management[17].

Nevertheless, there is scant evidence concerning the impact of vitamin D on PBMC secretions and their antibacterial efficacy against multidrug-resistant Acinetobacter baumannii obtained from chronic wounds. The objective of this in vitro study was to examine the influence of vitamin D on PBMC secretions and to assess their immunomodulatory and antibacterial properties against Acinetobacter baumannii, focusing specifically on cytokine production, bacterial growth inhibition, and biofilm formation.

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Materials and Methods

Ethics and consent

The study was approved by the Committee of College of Biotechnology, Al-Nahrain University No REC.COB/1204/15 dated on April 12, 2026. All donors were asked for their permission to involve them in the study with a written signed consent. The procedures followed according to the Helsinki Declaration of 1975 and further amendments.

Cell culture techniques

To reduce the risk of contamination and infection, all cell culture experiments were conducted in Class II cabinets. All of the cells were then incubated at standard culture conditions (SCC) in a humidified incubator set to 37°C, 20% O2, and 5% CO2. Unless otherwise specified, all culture media and solutions used in this investigation were purchased from (Capricorn/Germany). Peripheral blood mononuclear cells (PBMCs) were cultivated using Roswell Park Memorial Institute Medium (RPMI-1640). These media were supplemented with 100 µg/ml streptomycin (PS), 100 IU/ml penicillin, 4% of 200 mM L-glutamine, and 10% fetal bovine serum (FBS). Lymphocytes were cultivated using LymphoPrime complete media.

Study design and sample collection:

Peripheral blood (N=5 representing five biological replicates) was extracted using a tiny needle from both patients who had recently been in an accident and healthy volunteers of both sexes aged 15–30 years were included in the study. Smokers and individuals with chronic diseases were excluded. After that, the blood was kept in tubes with heparin to stop it from clotting. Ten milliliters of blood and ten milliliters of lymphocyte separation solution (density 1.077 g/ml) were added to a sterile conical tube, and the combination was centrifuged at 3500 rpm for fifteen minutes. After the PBMCs were banded in the interface between the gradient medium and plasma, they were collected using a pipette and moved into a fresh, sterile conical tube. Ten milliliters of PBS were then added, and the tube was centrifuged for ten minutes at 3000 rpm. After discarding the supernatant, the washing process was carried out three times. After being resuspended in 1 ml of RPMI-1640, the cell pellet was split into two culture flasks (T75 cm) and counted using a Mindray device. Twelve milliliters of lymphoPrime complete medium were added to the first flask which supports the growth of lymphocytes, and twelve milliliters of RPMI-1640 were added to the second flask which supports the growth of monocytes and macrophages. After counting the cells, a T75 flask was filled with 2×10^6 cells/ml. To make a cell suspension, ten milliliters of RPMI-1640 medium (for Monocytes) and ten milliliters of LymphoPrime complete medium (for lymphocytes) were added to the cells incubated at SCC. For untreated cells, after 72h of incubation, culture supernatants were collected and centrifuged at 3000 rpm for 5 min to remove cellular debris, filtered through a 0.22 µm membrane filter and kept at −80°C until further analysis.

In the vitamin D-treated group, monocyte and lymphocyte cultures were administered vitamin D (Mindary / China) at a final concentration of 20 ng/mL after 48 hours of incubation and were then incubated for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. Untreated cultures were sustained for the identical total incubation duration and functioned as controls.

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The Isolation and identification of bacteria from chronic wounds

One hundred patients with chronic wounds who gave written consent were included in this study. Between October 2025 and January 2026, samples were collected from Al-Yarmok Hospital and Baghdad Medical City. Samples from a chronic wound were collected using two sterile collection swabs that contained Amie's transport medium. After that, one swab was streaked on MacConkey agar and the other on blood agar. Suspected colonies were initially identified using colony morphology, Gram staining, catalase, and oxidase assays. The definitive identification of *Acinetobacter baumannii* was validated using the VITEK 2 automated identification system. For every assay, all bacterial isolates were grown to a density that roughly corresponds to a 0.5 MacFarland standard absorbance at 600 nm.

Testing antibacterial roles of PBMCs secretions on *A. baumanii*

Mueller Hinton agar was used to cultivate bacterial samples that were identified as *Acinetobacter baumannii* in order to evaluate their susceptibility or resistance to the medications ampicillin, imipenem, and vancomycin. The ability of PBMC secretions to inhibit bacterial growth was investigated. After soaking in PBMCs secretions for 15 minutes, a sterile disc the same size as antibiotic discs were placed on Mueller-Hinton agar-cultivated *Acinetobacter baumannii*, which were subsequently incubated overnight at 37 degrees Celsius. The inhibition zones were measured and compared to those of antibiotics after the results were evaluated. Biofilm formation quantitative microtiter plate experiments were performed using the protocol described by Thummeepak (2016) with some adjustments. Sterile 96-well polystyrene microtiter plates were filled with 100 µl of the bacterial suspension. For every treatment group, three duplicate wells were used in the test. Bacterial control wells, medium control wells, monocyte secretions before and after treatment, and lymphocyte secretions before and after treatment made up the experimental groups. For eighteen hours, the plates were maintained at 37°C. Following the incubation period, any non-adherent cells were eliminated by gently removing the planktonic cultures and rinsing the wells three times with phosphate-buffered saline (PBS). After being exposed to absolute methanol for ten minutes, the attached bacterial cells were stained for fifteen minutes using a 0.4% crystal violet solution. After rinsing the wells three times with sterile distilled water to remove any remaining stain, the plates were allowed to air dry. The bound crystal violet was then dissolved by adding 250 µl of 33% acetic acid to each well for 15 minutes. A microplate reader was used to measure the stained adherent bacteria's optical density (OD) at a wavelength of 595 nm. Bacterial adhesion and biofilm formation were inferred from the obtained optical density (OD) values.

Enzymes-linked immunosorbent assay (ELISA)

The levels of IL-6, IL-1β, and IFN-γ in culture supernatants were measured using commercial Quantikine ELISA kits (R&D Systems/Bio-Techne, Minneapolis, MN, USA) in accordance with the manufacturer's guidelines. The utilized kits were: Human IL-6 Quantikine ELISA Kit (Catalog No. D6050), Human IL-1β Quantikine ELISA Kit (Catalog No. DLB50), and Human IFN-γ Quantikine ELISA Kit (Catalog No. DIF50C). In summary, standards and samples were introduced to pre-coated wells, thereafter incubated with the detection antibody and streptavidin-HRP. Following the washing procedure, TMB substrate was introduced, the reaction was terminated with sulfuric acid, and absorbance was quantified at 450 nm with wavelength correction at 570 nm. Cytokine concentrations were determined from standard curves created with serially diluted standards. ANOVA was used to conduct the statistical analysis.

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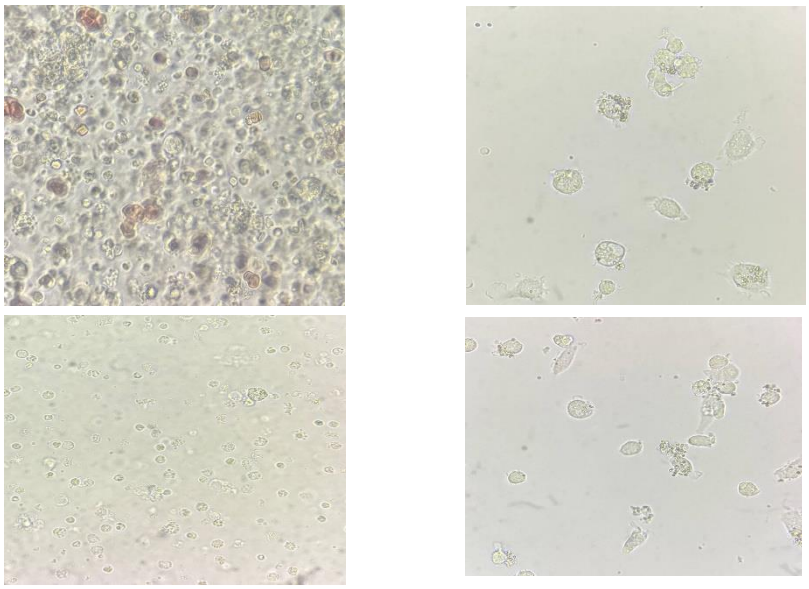
Statistical Analysis

Statistical differences among groups were evaluated using Analysis of Variance (ANOVA). Data are expressed as the mean ± standard error of the mean (SEM), and results were considered statistically significant at $p \leq 0.05$. Data analysis was performed using GraphPad Prism version 6.0 was used for data analysis

Results and Discussion

PMNCs and lymphocytes characteristics and secretions

The buffy cot gave a total cell count of 5×10^6 cell/ml. PMNCs grown in RPMI-1640 were able to adhere to culture flask after 7 days and contained mixed populations of cells depending on cell morphology. One population were round cells which are monocyte and the other population were star like-shape which are macrophage. On the other hand, cells grown in LymphoPrime media were round cells which are lymphocytes as shown in (figure 1).



(a)Macrophage and monocytes (20x) (b)lymphocytes (20x)

Figure 1. PMNCs and lymphocytes isolated from human peripheral blood. (a)mixed populations of monocyte macrophage at 20x magnification. (b)lymphocyte at 20x magnification.

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ELISA results that treatment with vitamin D has reduced the concentration of pro inflammatory cytokines. As the concentration of IL-6 has reduced from 585 pg/ml to 135 pg/ml. while IL-1B levels declined from 185 pg/ml to 135 pg/ml as well as IFN-Y concentration decreased from 440 pg/ml to 280 pg/ml after treatment with vitamin D. which indicate that vitamin D considered as anti- inflammatory and immunomodulatory vitamin by downregulating cytokine production (figure 2).

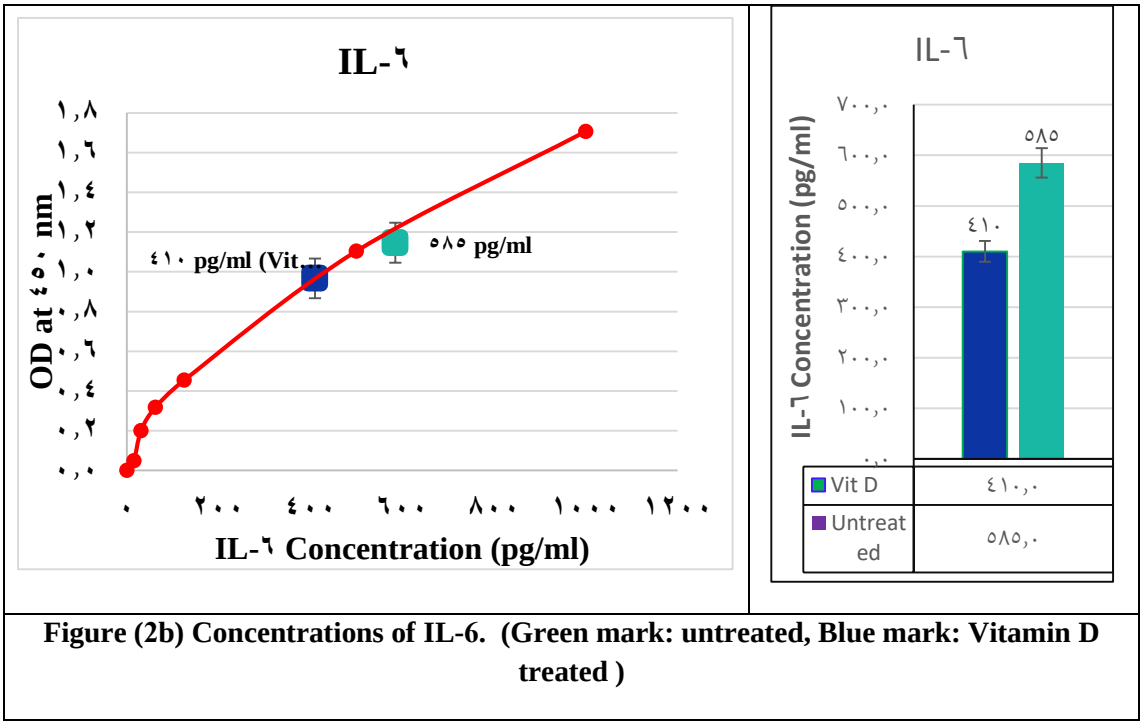

Figure (2b) Concentrations of IL-6. (Green mark: untreated, Blue mark: Vitamin D treated)

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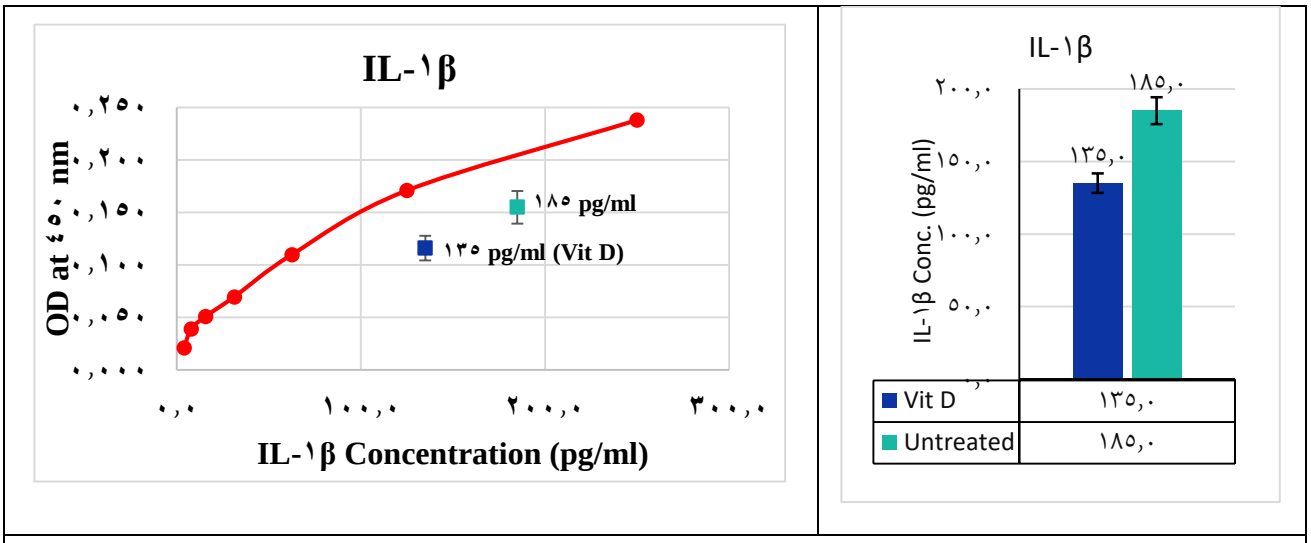

Figure (2d) Concentrations of IL-1β (Green mark: untreated, Blue mark: Vitamin D treated)

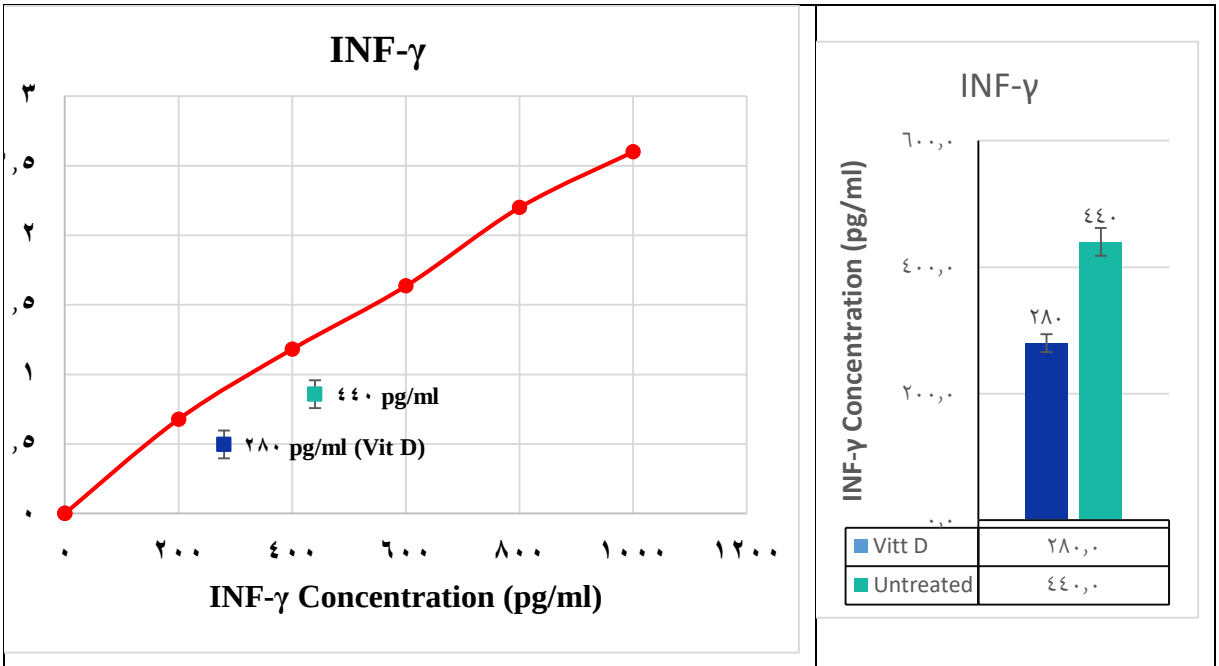

Figure (2f) Concentrations of INF-γ (Green mark: untreated, Blue mark: Vitamin D treated)

Figure 2: ELISA analysis of PBMCs secretions.

(a) Figure (2a) IL-6 Standard curve (b) Concentrations of IL-6 (Green mark: untreated, Blue mark: Vitamin D treated) (c) IL-1β Standard curve (d) Concentrations of IL-1β (Green mark: untreated, Blue mark:

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Vitamin D treated) (e) INF-γ Standard curve. (f) Concentrations of INF-γ (Green mark: untreated, Blue mark: Vitamin D treated)

Characteristics of *A. baumanii*

Acinetobacter baumannii was found in 40 isolates out of 100 samples. Small to medium-sized, smooth, round, convex, opaque colonies with a pale white to grayish appearance were observed in colony morphology. The colonies appeared to be non-pigmented and had a somewhat damp surface. *A. baumannii* generated smooth, creamy-white colonies on blood agar without total hemolysis, exhibiting non-hemolytic growth surrounding the colonies (Figure 3 A). The organism's capacity to endure aerobic conditions and adjust to various environmental stresses allowed it to grow well on blood agar. The VITEK 2 system was used to test a number of biochemical reactions (fig. 3B), which showed the bacterial traits and enzymatic activity of the isolated samples. The isolates exhibited negative results for the oxidase test, urease hydrolysis, indole production, and lactose fermentation, but positive results for glucose oxidation, citrate utilization, and catalase activity. These results align with the typical biochemical and phenotypic traits of *Acinetobacter baumannii*.

(a) *Acinetobacter baumannii* on blood agar

Identification Information		Analysis Time:	7.97 hours	Status:	Final
Selected Organism		93% Probability	Acinetobacter baumannii complex		
		Bionumber:	0243051101500352		
ID Analysis Messages					

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	+	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	+	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

(b) VITEK 2 system characterization

Figure 3: *Acinetobacter baumannii characterization*
(a) *Acinetobacter baumannii on blood agar* show white colonies.
(b) VITEK 2 system characterization

PMNCs and lymphocyte secretions inhibit growth of *A. baumannii*

The activity of immune cells seemed to be affected differently by vitamin D. Following vitamin D treatment, monocyte secretions exhibited enhanced antibacterial activity against A. baumannii, while T-lymphocyte secretions showed decreased antibacterial activity. The immunomodulatory function of vitamin D and the different immunological roles of innate and adaptive immune cells may be connected to this

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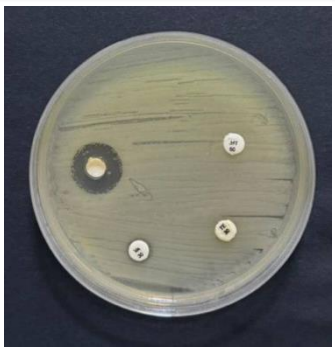
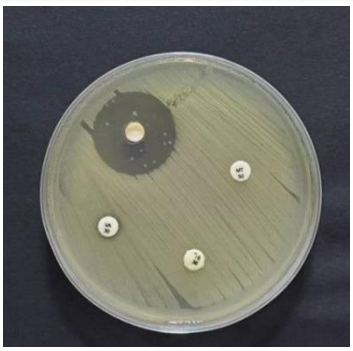
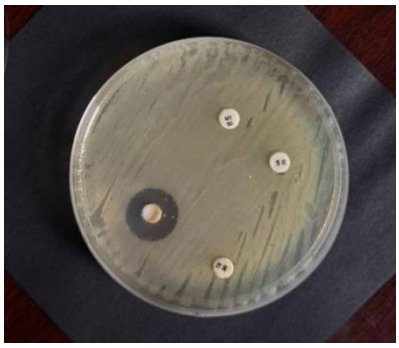
discrepancy. The findings demonstrated that in the bacterial control group, Acinetobacter baumannii produced a robust biofilm. When compared to the control, monocyte and lymphocyte secretions decreased the formation of biofilms. While lymphocyte secretions maintained low biofilm formation both before and after vitamin D exposure, monocyte secretions following vitamin D therapy displayed lower OD595 values than before treatment.



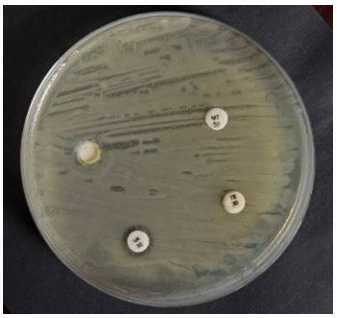
(a) Monocytes secretion pre treatment



b) monocytes secretion pro treatment



(c) Lymphocytes secretions pre treatment

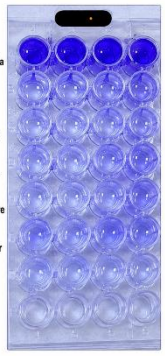


(d) Lymphocytes secretionspre treatment

Biofilm Formation Assay (Crystal Violet Staining)

Group	Column 4 (OD595)	Column 5 (OD595)	Column 6 (OD595)	Mean (OD595)
A. Bacteria	0.713	0.444	0.393	0.517
B. RPMI	0.679	0.284	0.266	0.410
C. Monocyte before	0.600	0.705	0.740	0.682
D. Monocyte after	0.441	0.738	0.710	0.630
E. Lymphocyte before	0.063	0.057	0.053	0.058
F. Lymphocyte after	0.049	0.054	0.053	0.052

Note: Lower OD595 values indicate lower biofilm formation.



(e)Representative image of biofilm plate

Figure 4: Representative images of antibacterial activity of PMNCs against *S. A.baumannii*
(a)Monocytes secretion pre treatment. (b) monocytes secretion pro treatment. (c) Lymphocytes secretions pre treatment (d) Lymphocytes secretions pre treatment (e) Microtiter plate contained biofilm of A. baumannii treated with TSB, PBMMNCS secretions before and after treatment.

The current investigation indicated that vitamin D administration correlated with reduced release of IL-1β, IL-6, and IFN-γ from PBMC-derived cells. The results indicate that vitamin D may affect inflammatory responses within the experimental parameters of this investigation. Previous reports have indicated a correlation between vitamin D and the control of pro-inflammatory cytokine production[18]. Vitamin D binding to vitamin D receptors (VDRs) expressed on monocytes, macrophages, and lymphocytes is known to activate a variety of immunomodulatory and antimicrobial pathways [12]. By stimulating the transcription of antimicrobial peptides, boosting phagocytic activity, and regulating the generation of

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inflammatory cytokines, Previous studies suggest VDR signaling activation enhances host defense against bacterial infection [14]. These secretions demonstrated a dual mechanism of action that is crucial to the pathogenicity and persistence of A. baumannii infections by inhibiting bacterial growth and biofilm formation. Cytokines, one of the vital immune substances, play a key role in the activation of the immune system [19]. IL-1β is one of the primary pro-inflammatory cytokines that stimulates leukocytes and increases inflammatory reactions during wound infection. Persistent elevation of IL-1β has been associated with tissue damage and delayed wound healing in chronic inflammatory situations Therefore, the reduction in IL-1β observed in the present study may contribute to a microenvironment more favorable for wound healing inflammatory tissue damage [20]. Similarly, reduced IL-6 release after vitamin D therapy may be due to inhibition of chronic inflammatory signaling pathways. IL-6 controls both acute and chronic inflammatory responses, and persistently high IL-6 has been linked to prolonged immune activation and poor wound healing. Previous research has shown that vitamin D inhibits IL-6 production by controlling inflammatory transcription pathways and NF-κB activation. Therefore, the reduction in IL-6 observed in this study may indicate that vitamin D has anti-inflammatory qualities and that immunological homeostasis has been restored in the wound microenvironment [21]. The current results also indicated a decrease in IFN-γ subsequent to vitamin D delivery. While IFN-γ is essential for antibacterial immunity, its excessive or prolonged production may lead to chronic inflammation and tissue damage. The decrease in IFN-γ observed after vitamin D administration should not be construed as immunosuppression. Vitamin D regulates Th1-mediated immunity, so curtailing excessive inflammatory activity while maintaining vital immunological activities[22]. Consequently, the decreased IFN-γ levels observed in the present study may reflect immune regulation rather than impairment of antibacterial defense mechanisms[15] . Besides IL-1β, IL-6, and IFN-γ, secretions from PBMCs are recognized to encompass an array of cytokines, chemokines, growth factors, and antimicrobial agents. The current investigation concentrated exclusively on certain inflammatory cytokines and did not delineate the entire secretory profile of monocyte- and lymphocyte-derived secretions.

This interpretation is particularly relevant in chronic wounds, where persistent inflammation contributes to delayed healing and tissue injury [23]. In addition to its effects on cytokine production, PBMC secretions exhibited antibacterial activity against A. baumannii and reduced biofilm formation. These findings are particularly relevant because biofilm development is considered a major factor contributing to chronic wound persistence and antimicrobial resistance[24-25].

The documented antibacterial and antibiofilm properties indicate that vitamin D-modulated PBMC secretions maintained biological efficacy under the in vitro conditions employed in this research. Prior research has shown that secretions generated from PBMCs encompass cytokines, growth factors, and various immunological mediators that influence their biological effects[١٦]. The identification of cytokines, along with the suppression of A. baumannii proliferation and biofilm development, indicates that the obtained secretions contained functionally active immune mediators throughout the culture period. Nevertheless, the particular mediators accountable for these effects were not ascertained in the current investigation.

Further in vivo and clinical studies are required to determine the therapeutic importance of these findings. Several constraints must be recognized while evaluating the present results. The donor sample size was restricted (N = 5), perhaps limiting the generalizability of the results. Furthermore, molecular characterization of immune responses was not performed, hence the mechanisms regulating cytokines were not specifically investigated. The lack of data for antimicrobial peptides such as cathelicidin (LL-37) and β-defensins hindered the identification of the specific mediators accountable for the observed antibacterial effect. The study was performed solely in vitro, without in vivo confirmation. Thus, although the data suggest that vitamin D-modulated PBMC secretions may contribute to immunological modulation, bacterial growth inhibition, and biofilm reduction, these findings should be considered preliminary and not interpreted as evidence of therapeutic efficacy. Further research employing larger sample sizes, molecular analyses, antimicrobial peptide quantification, and animal or clinical models is essential to corroborate these findings and to evaluate the potential role of vitamin D in the treatment of chronic wounds and multidrug-resistant bacterial infections.

Conclusion

This study concluded that vitamin D administration correlated with decreased release of the pro-inflammatory cytokines IL-6, IL-1β, and IFN-γ from PBMC-derived cells in vitro. Furthermore, PBMC secretions demonstrated antibacterial efficacy against Acinetobacter baumannii and diminished biofilm formation, a critical virulence characteristic linked to bacterial persistence and antimicrobial resistance. The results indicate that vitamin D may affect the immunological and antibacterial characteristics of secretions generated from PBMCs under the experimental conditions employed in this investigation. Nonetheless, the fundamental molecular pathways were not examined, and the results are constrained by the in vitro method. Consequently, additional preclinical and clinical investigations are necessary to validate these findings and to enhance the understanding of the possible influence of vitamin D-modulated PBMC secretions in chronic wound infections induced by multidrug-resistant bacteria.

Acknowledgments

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Author’s Declaration

-The authors declare no conflict of interest.

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