Comparison the Antibacterial Activity of Vitamin D2 and D3

Intesar N. Khelkal
Department of Biology, College of Science, Al-Mustansiriyah University,Iraq

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ABSTRACT

An attempt has been made to determine the antimicrobial activity of vitamin D; D3 & D2 against clinical bacterial isolates as well as perform a comparative analytical study between the effects of both forms of vitamin.

The ability of vitamin D (both D2 ergocalciferol& D3 cholecalciferol) to inhibit bacterial growth of some clinical isolates have been tested . Forty - three pathogenic bacterial isolates (Gr+ ,Gr-) have been identified from fifty - five specimen was collected from different sources ; 24 urine, 17 sputum , 9 blood , 5 skin at Al-Kindey hospital for a period of two months . Antibiotic sensitivity was carried out towards 12 different antibiotic discs. The most resistant isolates have been chosen to be tested in the study.

Two bacterial suspensions of the selected isolates have been prepared; the first was adjusted to McFarland standard No. 0.5 (1×10^{8} CFU /mL); the second = 1×10^{10} CFU /mL. Three concentrations of both vitamins have been prepared; 50,000, 70,000 and 90,000 IU/mL as well as the control (solvent only). Antibacterial activity has been examined by using agar diffusion (pore plating method) to determine the most effective concentration among the three concentrations of the two forms of vitamin D.

Results were suggesting the important role of vitamin D specially D3 as antibacterial agent .The third concentration (90,000) IU/mL was causing the largest inhibition zone with all tested isolates even with the high turbidity culture (10^{10}CFU/mL) ,followed by the second one (70,000) IU/mL, the lower inhibitor concentration was (50,000) IU/mL. Significant differences have been appeared among the measurements of the diameters of inhibition zones towards three vitamin concentrations when compared one to another and to control.

الخلاصة

أجريت محاولة لأختبار قدرة فيتامين د بشكليه : أركوكالسيفيرول د2 وكوليكالسيفيرول د3 على تثبيط نمو عزلات بكتيرية مرضية وعمل مقارنة أحصائية بين الفعل التثبيطي لشكلي الفيتامين د2 ود3.

أستخدمت في الدراسة الحالية(43) عزلة بكتيرية مرضية(Gram-positive and Gram-negative) جمعت من (55)عينة لحالات سريرية مختلفة :24 من الأدرار ،17من القشع،9 من الدم ، 5 من الجلد من مستشفى الكندي التعليمي ولمدة شهرين.

حددت مدى حساسية ومقاومة العزلات تجاه 12 من المضادات الحيوية بأختبار كيربي- باور وأختيرت ست عزلات هي ألأكثر مقاومة منها للكشف عن تاثيرالتراكيز المختلفة من الفيتامين.

حضرت ثلاثة تراكيز من فيتامين د:50000 و70000 و 90000 وحدة دولية/ مل وأختبر فعلها ضد تركيزين لكل عزلة: 1010 و 810 CFU /mL. اختبرت الفاعلية ضد البكتيرية بأستخدام الأنتشار في الأكار بطريقة الثقوب لتحديد التركيز ألأكفأ من بين التراكيز الثلاثة لكلا شكلي الفيتامين.

أظهرت النتائج قدرة عالية لفيتامين د ولاسيما د3على تثبيط نمو العزلات قيد الدراسة وذلك بالتركيز 9000 وحدة دولية/مل الذي سبب ظهور منطقة تثبيط واسعة مع كل العزلات قيد الأختبار حتى بتراكيزها العالية(1010 CFU /mL) ، تبعه التركيز الثاني 70000 وحده دولية/مل ،ثم التركيز الأقل 50000 وحدة دولية / مل. وتبين وجود فروقات معنوية ضمن قياسات أقطار مناطق التثبيط تجاه تراكيز الفيتامين الثلاثة عند مقارنتها مع بعضها البعض ومع السيطرة.

INTRODUCTION

Vitamins are organic substances that are required in small amounts for maintenance and growth, but cannot be manufactured by the human body. [1] The Vitamin D appears to have systemic antimicrobial effects that may be crucial in a variety of both acute and chronic illness. Vita [2] min D is actually a fat-soluble prohormone steroid that has endocrine, paracrine and autocrine functions , it also improves survival in acute illness by boosting innate immunity [3].

Although there are many therapeutic agents for treating them but infectious diseases are one of the main causes of morbidity-mortality in the world and prolonged antibiotic therapy induce bacterial-resistance because some bacteria have developed ways to circumvent the effects of antibiotics. Therefore, antibiotic resistance can be considered as a serious threat for health, and an international approach to its management is required, thus, new drugs have been developed for control of bacterial resistance [2,3].

The role of vitamins in the combat of disease is interpreted as acting by modulating the immune response of an infected host. [4] It had been hypothesized that some vitamins may influence the bacterial growth, particularly Mycobacteria. Vitamins A and D cause

dose-dependent inhibition of all three mycobacterial species[5].
The current use of antimicrobials costs billions of dollars and the overuse of antibiotics contributes to resistant organisms such as methicillin resistant *S.aureus* [6]. Vitamin D using could potentially reduce inappropriate antibiotic prescribing and boost therapeutic response when combined with appropriate antibiotic use .Thus it modulates the antimicrobial response [7] .
Recent studies revealed the importance of the vitamin D-dependent generation of antimicrobial peptides in human host defense against *Mycobacterium tuberculosis* [8,9] .
Gram-positive bacteria, invasive pneumococcal disease, meningococcal disease and group A streptococcal disease are more common when vitamin D levels are low, raising the possibility that pharmacological doses of vitamin D could be an effective adjuvant therapy [10] Ergocalciferol is the chemical name of vitamin D_2, molecular formula: $C_{28}H_{44}O$, molecular weight: 396.64836 g/mol, melting point 116.5°C. Cholecalciferol is the chemical name of vitamin D3. Chemical & Physical properties of vitamin D3 are: The molecular formula for cholecalciferol ($C_{27}H_{44}O$). Odorless [11]. Molecular-weight 384.63766g/mol. Solubility; Sol in the usual org solvents .Melting Point; 84-85 deg C[12] .

MATERIALS AND METHODS

Materials

1- Bacterial isolates: (55) clinical samples collected from different sites; 24 specimens from urine, 17 sputum sample, 9 samples of blood, 5 swabs of skin infections, (43) were positive for bacteriological culture.
2- Vitamins: D3 & D2 have been provided from Xian lypha Biotechnology company (China) with concentration; 100,000 IU/gm for each.
3- Culture media; nutrient agar, MacConkey agar,brain –heart infusion broth & agar,blood agar,Mueller-Hinton agar were provided from oxoid (England), Himedia (India), Fluka (Germany).
4- Antibiotics discs were provided by Bioanalyse (Turkey).
5- DMSO; Dimethyl sulfoxide which has been used in dissolving the vitamin powder as well as a control (only DMSO without vitamin). DMSO was the dissolvent factor or dilute solution because (DMSO) is an organosulfur compound with the formula $(CH_3)_2SO$. This colorless liquid is an important polarsolvent that dissolves both polar and nonpolar compounds. It has a relatively high melting point. Vitamin D derivatives water-insoluble but fat soluble [13].

Methods

This study has been conducted in Microbiology labs. Biology Department/ College of Science/ Al-Mustansiriyah University

Samples Collection

1-Isolation and Identification: All bacterial isolates used in this study were clinical Gram positive and negative bacteria. Clinical samples were cultured on its suitable media , after incubation period, identification have been carried out by routine morphological & standard biochemical tests, the diagnosis was confirmed by Vitek 2 system provided by BioMereurix (France).

2-Vitamins concentrations were prepared according to (14): 0.5 gram of the vitamin powder was added to 1mL of the organic solvent, mixing well to obtain 50,000 IU/mL, 0.7, 0.9 gram was added to 1 ml of the solvent to get 70,000 & 90,000 IU/mL respectively.

3-Two bacterial suspensions were prepared; 10^8, 10^{10} (CFU /mL). These concentrations were standardized by UV spectrophotometer (SP-3000nano/OPTIMA).

4- Antibacterial activity of the three concentrations of both vitamins were estimated by determining the diameters of inhibition zones around holes which containing the tested vitamins by pore plating method in agar, 100 ml of the mixture prepared in 2 was added to each pore beside the control pore (solvent only). Each experiment has been done in triplicate.

5- Antibiotic sensitivity test have been carried out according to (15) using Kirby-Bauer disc diffusion method.
6-**Statistical analysis** have been done according to[16,17] .

RESULTS AND DISCUSSION

The present study have dealt with the bacteriostatic activity of vitamin D forms ; D2 &D3 and aimed to determine if this vitamin have the ability to inhibit bacterial growth or not also to detect which one (with certain concentration) have the high activity against clinical isolates.
Out of 43 clinical isolates ;28 were Gram-negative , *K.pneumoniae* was the predominant organism (9) isolates ; followed by *E.coli*(8)then *Ps.aeruginosa*(5). Gram –positive isolates were 17 distributed as ; 9 isolates belonged to *S. aureus* while 5 for *Strep.pneumoniae* and 2 for Strep.pyogenes as shown in Table 1;

Table 1 Results of Identification

Sample	No. of clinical samples (No. of isolates)	Identification

Urine	24 (19)	(7) *E.coli*, (4) *K.pneumoniae*, (3) *S.aureus* (3) *P.mirabilis*. (2) mixedinfection:1*(S.aureus+Ps.aeruginosa)* and 1(*E.coli +Ps.aeruginosa*)
Sputum	17 (15)	(5) *Strep.pneumoniae*, (5) *K.pneumoniae*, (2) *Strep.pyogenes*, (2) *S.aureus*, (1) *Haemophilusinfluenza*
Blood	9 (4)	(2) *A.baummanii*, (1) *Ps.aeruginosa*, (1) *S.aureus*
Skin infections	5 (5)	(2) *S.aureus*, (2) *Ps.aeruginosa*, (1) *S.warneri*
	55(43)	**Identified 43**

Antibiotic Sensitivity: Twelve different antibiotic discs were used : levofloxacin 5µg, ciprofloxacin 10µg, cefotaxime 30µg, amoxicillin-clavulan acid 30µg, imipenem 10µg, aztreonam 30µg, meropenem 10µg, azithromycin 15µg, chloramphenicol 30µg, nitrofurantoin 30µg, rifampicin 15µg, vancomycin 10µg. Bactrial resistance is one of the major causes of failure in the treatment of infectious diseases resulting in increased morbidity, it was observed from the present study that clinical bacterial isolates which were tested have showed high resistant to conventional antibiotics.

E. coli was sensitive to levofloxacin and ciprofloxacin and nitrofurantoin. *K. pneumoniae* was sensitive to ciprofloxacin, levofloxacin, chloramphenicol and imipenem,*Ps.aeruginosa* was sensitive to ciprofloxacin and meropenem while*S. aureus* was sensitive to rifampicin, levofloxacin, and vancomycin. These results were compatible with other studies [18,19] that tested antibacterial sensitivity towards Gr+ & Gr-isolates; rifampicin, levofloxacin & vancomycin were the most effective antibacterial agents against Gr+ bacterial isolates in our study whereas ciprofloxacin & levofloxacin were the most effective towards Gr-isolates. Six bacterial isolates have been chosen to detect the vitaminefficacy ; SaS, StpS , EcU, AbB, KpS, PsB which represent highly multi-drug resistant isolates; *S.aureus*(sputum), *Strep.pneumoniae* (sputum), *E.coli* (urine), *A.baumannii* (blood), *K.pneumoniae* (sputum), *Ps.aeruginosa* (blood) respectively.

Tables 2, 3, 4, 5 showed the measurement of the diameters of inhibition zones that had been resulted from treatment of different clinical isolates with vitamin D:

Table 2 Diameters of Inhibition Zones of Tested Bacterial Isolates (10^{10} CFU/mL)Against Different Concentrations of Vitamin D3

Vitamin conc.(IU/mL)	**50000**	**70000**	**90000**
Isolate	**Diameter of Inhibition Zone(mm)**		
SaS	15	19	29
StpS	17	22	26
EcU	10	13	15
AbB	12	16	17
PsB	11	14	19
KpS	7	10	18

Mean	12	15.667	20.667
SD	3.578	4.32	5.538
Comparison	Diff of Means	t.test	P<0.05
90000vs50000	8.667	3.298	0.015 S
90000vs70000	5	1.903	0.147 NS
70000vs50000	3.667	1.396	0.183 NS

SaS;*Staph.aureus* (sputum)-**StpS**;*Strep.pneumoniae* (sputum)-**EcU**;*Escerichia coli* (urine)-**AbB**;*Acinetobacter baumannii* (blood)-**KpS**;*klebsiella pneumoniae* (sputum) **PsB**;*Pseudomonas aeruginosa* (blood)

The results in Table 2 and Figure 1 were shown clearly the ability of vitamin D3 to inhibits the bacterial growth of both Gr+& Gr- isolates , the third concentration 90,000 IU/mL was the most effective one particularly towards SaS & StpS because of the large inhibition zone that result (29,26 mm) respectively,whereas 70,000 IU/mL caused less inhibition zone (22mm) with StpS isolate ,the bacterial suspension was in conc.of (10^{10} CFU/mL). Small inhibition zone was reported after treatment KpS with the first and second vit.concentrations ,those caused (7,10 mm) while EcU has been showed (10 mm) with 50000 IU/mL as shown in Figure 1:

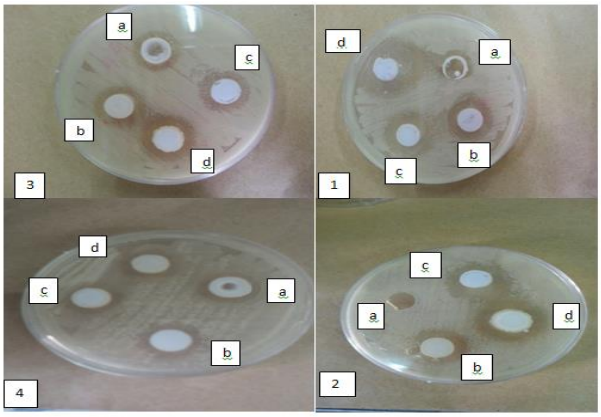

Figure 1 Effect of vitamin D3 on bacterial growth (10 ^{10} CFU/mL), 1. StpS, 2. SaS, 3.EcU, 4.AbB, -Control, b-50000, c-70000, d-90000 control; solvent only (DMSO) except for 2; control is normal saline.

Significant differences have been appeared when compared the effect of vit.concentration (90000) with (50000)IU/mL in significance level (P<0.050) by using diff. of means and t-test which was equall to 3.594 so it was considered to be significant at 0.05.

Noticeable inhibition have been observed around control holes which contains the solvent only (DMSO) as in Figure 1 , it exhibits a weak antibacterial effect may be because of the sulfur in its chemical structure it may possess synergistic effect within vitamin D and cooperative behavior in inhibition bacterial growth, the control in Figure 1(2) is normal saline - one of triplicate of tested SaS - instead of DMSO , no inhibition zone was recorded.

Table 3 Diameters of Inhibition Zones of Tested Bacterial Isolates (10^8 CFU/mL) Against Different Concentrations of Vitamin D3

Vitamin conc.(IU/mL)	50000	70000	90000
Isolate	Diameter of Inhibition Zone(mm)		
SaS	18	21	29
StpS	20	23	29
EcU	13	15	18
AbB	14	17	21
PsB	11	14	17
KpS	9	12	21
Mean	14.167	17	22.5
SD	4.167	4.243	5.282
Comparison	Diff of Means	t.test	P<0.05
90000vs50000	47	3.594	0.030 S
90000vs70000	28	2.141	0.284 N.S
70000vs50000	19	1.453	0.56 N.S

Noticeable increases in the diameters of inhibition zone have appeared when we tested 10^8 CFU/mL as a bacterial suspension with the different concentrations of vit.D3.Highest measurements (29mm) have recorded with SaS & StpS when treated with 90000 IU/mL while (9,11mm) were observed for KpS,PsB respectively with 5000 IU/mL (Table 3 & Figure 1). Also significant differences were documented between 90000 & 50000 IU/mL with 0.030 less than (P<0.050).

These results indicated to considerable activity of vit.D towards Gr+bacteria (SaS & StpS) clinically isolated from sputum compared with Gr- (EcU,AbB,PsB,KpS) multidrug-resistant clinical isolates ,that may be attributed to the complexity of their outer membrane including lipopolysaccharide structure.

Invitro study of (14) proved that vitamin D_3 has inhibitory activity on strains of *S. aureus, Strep. pyogens, K. pneumoniae, E. coli* and *Candida spp*. in the presence of 50,000–90,000 IU/mL of vitamin D_3, the organisms were killed or demonstrated marked inhibited growth . These results were agreed with the present study in particular with the results of vitamin D3 (except for *Candida spp*.which didn't tested in our study).

Table 4 Diameters of Inhibition Zones of Tested Bacterial Isolates (10^{10} CFU/mL) Against Different Concentrations of Vitamin D2

Vitamin conc.(IU/mL)	50000	70000	90000
Isolate	Diameter of Inhibition Zone(mm)		
SaS	13	15	20
StpS	14	16	18
EcU	11	13	18
AbB	10	13	17
PsB	10	14	17
KpS	6	11	18
Mean	10.667	13.667	18
SD	2.805	1.751	1.095
Comparison	Diff of Means	t.test	P<0.05
90000vs50000	7.333	9.242	0.010 S
90000vs70000	4.333	5.461	0.010 S
70000vs50000	3	3.781	0.040 S

As in Table 4 and Figure 2 there were significant differences among the results according to differences of means and t-test (P<0.050), high inhibition have appeared after treatment with 90,000 IU/mL of vit.D2 with the SaS (20 mm) followed by StpS , EcU & KpS(18 mm)for each. The lowest zone was for KpS (6 mm) with 50,000 IU/mL . Apparently from these results vit.D2 have good activity as bacteriostatic for pathogenic bacteria specially towards Gr+ isolates but when compared to vit.D3,we observed the last have higher inhibitory activity that may be attributed to distinguished chemical structure ,less weight, lower melting point& high solubility in organic solvents.

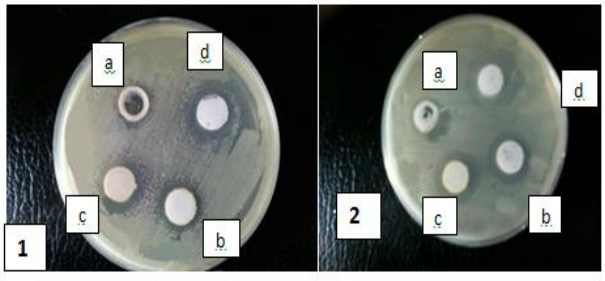

Figure 2 Effect of different concentrations of vitamin D2 on bacterial growth (10^{10} CFU/mL): 1. SaS, 2. PsB a-Control, b-50000, c-70000, d-90000 control; solvent only.

Table 5 Diameters of Inhibition Zones of Tested Bacterial Isolates (10^8 CFU/mL) Against Different Concentrations of Vitamin D2

Vitamin conc.(IU/mL)	50000	70000	90000
Isolate	Diameter of Inhibition Zone(mm)		
SaS	16	19	23
StpS	17	20	23
EcU	14	16	21
AbB	13	15	19
PsB	14	17	22
KpS	9	10	23
Mean	13.83	16.16	21.83
SD	2.787	3.545	1.602
Comparison	Diff. of Means	t.test	P<0.05
90000vs50000	14	5.292	0.010 Yes
90000vs70000	7	2.646	0.147 No
70000vs50000	7	2.646	0.147 No

Results shown in Table 5 were indicated to efficient inhibition of pathogenic isolates by vit.D2 in 90000 IU/mL for SaS , StpS , PsB & KpS the inhibition zone was larger than what have seen in the previous table(4) because the bacterial suspension 10^8 CFU/mL lower than 10^{10} CFU/mL also there was significant differences between the effect of 90000 & 50000 IU/mL (0.010) less than P<0.050 as well as diff.of means & t-test were acceptable.

It is obvious from preceding results of statistical analysis that the efficiency of vit.D3 as bactericidal or bacteriostatic is higher than vit.D2 .

Vitamin D appeared to have systemic antimicrobial effects that may be important in a variety of both acute and chronic illness. Vitamin D may reduce the risk of infection through multiple mechanisms ,it has diverse and potent local and systemic activities such as enhanced production of anti-microbial peptides [2].

Invitro studies of the past 30 years have identified numerous mechanisms for the antibiotic effects of vitamin D in humans with induction of antimicrobial or bactericidal peptides being of greatest interest. Studies with other infectious conditions suggest that adequate vitamin D levels or supplementation with vitamin D may be important in reducing respiratory tract and vaginal infections [7].

Gram-positive bacteria, invasive pneumococcal disease, meningococcal disease and group a streptococcal disease are more common when vitamin D levels are low, raising the possibility that pharmacological doses of vitamin D could be an effective adjuvant therapy [10] .

Studies have found that vitamin D plays an important role in immune function via a several pathways, including enhancing the release of antimicrobial peptides in the skin, low serum vitamin D levels may increase the risk of nasal carriage of methicillin-resistant *S. aureus* (MRSA) so that vitamin D deficiency is associated with an increased risk of MRSA nasal carriage that further trials may be warranted to determine whether vitamin D supplementation decreases the risk of MRSA colonization [20,21].

More recent studies with pulmonary tuberculosis use much lower doses of vitamin D in combination with current antibiotic therapies and the findings are mixed [7].

Vitamin D stimulates the expression of potent antimicrobial peptides, such as cathelicidin and β-defensin 2 [22] which exist in neutrophils, monocytes, natural killer (NK) cells and epithelial cells lining the respiratory tract [23]. Macrophages, lymphocytes and monocytes have;Vitamin D-Receptors(VDRs) that, with 25 (OH) D stimulation, increase the expression of these antimicrobial peptides [24]. [25] noted a positive relationship between vitamin D levels and cathelicidin levels in acutely ill patients.

A double-blind randomized placebo-controlled trial involving young children in an inner- hospital in Kabul showed that the risk of a repeat episode of pneumonia within 90 days of supplementation of oral 100,000 IU of vitamin D_3 was lower in the intervention than in the placebo group. [26]

Inflammation resulting from the immune response targeting *Propionibacteriumacnes*(*P. acnes*)has a significant role in acne pathogenesis. In a recent study, it has been demonstrated that *P. acnes* is a potent inducer ofTh17, and that 1,25OH2D inhibits *P. acnes*-induced Th17 differentiation, and thereby could be considered as an effective tool in modulating acne ,that demonstrate the important role of vitamin D in skin infections [27] .

Results of [28] showed that Gram negative bacteria were more resistant to vitamin E effect than Gram positive bacteria, this resistance towards antibacterial substance may be related to lipopolysaccharides (LPS) in their outer membrane.

Experimental data of [29] suggested that quaternary amine group involved in the HPVB1 conjugate requires the hydrophobic region of the steroid in order to interact with some components of bacterial cell, disturbing the bacterial growth and to cause cell death, so the synthesis of cholic acid-derivate compounds that have antibacterial activity by increasing the permeability of the outer membrane of Gram negative bacteria have shown excellent results.

After progressive survey in literatures, we did not find any local *In vitro* study about antibacterial effect of vitamin D3 & D2 .More researchs are needed to determine the role of vitaminD and it's most effective bactericidal or/and bacteriaostatic concentration specially in experimental animals (*In vivo* study).As well as Investigating the possibility to prepare a combined drug from vitamin D3 or D2 & certain antibiotic.

We concluded from this study that vitamin D have potential activity towards clinical bacteria ,VD3 was

better inhibitor than VD2 even in high bacterial number of cells that may present promising results .
Supplementary studies may be required to establish vitamin D antibacterial activity and the possible mechanisms by which vitamin D may have a therapeutic role in managing a variety of infections particularly against pathogenic bacteria and development of potential therapeutic applications.

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