Comparison the Antibacterial Activity of Vitamin D2 and D3

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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 has been tested. Forty-three pathogenic bacterial isolates (Gr+, Gr-) have been identified from fifty-five specimens collected from different sources; 24 urine, 17 sputum, 9 blood, 5 skin at Al-Kinsey hospital for a period of two months. Antibiotic sensitivity was carried out towards 12 different antibiotics. The most resistant isolates have been chosen for testing 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). Antimicrobial 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.

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Results were suggesting the important role of vitamin D specially D3 as an 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 (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.

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. Vital2min 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 D2, molecular formula: \( \text{C}_{27}\text{H}_{44}\text{O} \), 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 (\( \text{C}_{27}\text{H}_{44}\text{O} \)), 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 JyphaBiotechnology company (China) with concentration; 100,000 IU/gm for each.

3-Culture media; nutrient agar, MacConkeyagar,brain – heart infusion broth & agar,bloodagar,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 dissolver factor or dilute solution because (DMSO) is an organosulfur compound with the formula (CH₃)₂SO.

This colorless liquid is an important polar solvent that dissolves both polar and nonpolar compounds. It has a relatively high melting point. Vitamin D derivatives are 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 BioMerieux (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; \( 10^6,10^9 \) (CFU/mL). These concentrations were standardized by UV spectrophotometer (SP-3000nano/OPTIMA ).

4- Anti-bacterial 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- Anti-biotic 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>No. of clinical samples (No. of isolates)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>24 (19)</td>
<td>(7) E. coli, (4) K. pneumoniae, (3) S. aureus, (3) P. mirabilis, (2) mixed infection: 1(S. aureus + P. aeruginosa) and 1(E. coli + P. aeruginosa)</td>
</tr>
<tr>
<td>Sputum</td>
<td>17 (15)</td>
<td>(5) S. pneumoniae, (5) K. pneumoniae, (2) S. aureus, (1) Haemophilus influenza</td>
</tr>
<tr>
<td>Blood</td>
<td>9 (4)</td>
<td>(2) A. baumannii, (1) P. aeruginosa, (1) S. aureus</td>
</tr>
<tr>
<td>Skin infections</td>
<td>5 (5)</td>
<td>(2) S. aureus, (2) P. aeruginosa, (1) S. warneri</td>
</tr>
<tr>
<td></td>
<td>55 (43)</td>
<td>Identified 43</td>
</tr>
</tbody>
</table>

Antibiotic Sensitivity: Twelve different antibiotic discs were used: levofloxacin 5µg, ciprofloxacin 10µg, ceftaxime 30µg, amoxicillin-clavulan acid 30µg, imipenem 10µg, aztreonam 30µg, meropenem 10µg, azithromycin 15 µg, chloramphenicol 30µg, nitrofurantoin 30 µg, rifampicin 15 µg, vancomycin 10µg. Bacterial 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, and meropenem. P. aeruginosa was sensitive to ciprofloxacin and vancomycin. These results were compatible with other studies [18, 19] that tested antibacterial sensitivity towards Gr+ & Gr- isolates. Rifampicin, levofloxacin, and 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 vitamin efficacy: S. aureus (sputum), S. pyogenes (sputum), E. coli (urine), A. baumannii (blood), K. pneumoniae (sputum), P. aeruginosa (blood) respectively.

Table 2. Diameters of Inhibition Zones of Tested Bacterial Isolates (10^9 CFU/mL) Against Different Concentrations of Vitamin D3

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Diameter of Inhibition Zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50000</td>
</tr>
<tr>
<td>SaS</td>
<td>15</td>
</tr>
<tr>
<td>StpS</td>
<td>17</td>
</tr>
<tr>
<td>EcU</td>
<td>10</td>
</tr>
<tr>
<td>AbB</td>
<td>12</td>
</tr>
<tr>
<td>PsB</td>
<td>11</td>
</tr>
<tr>
<td>KpS</td>
<td>7</td>
</tr>
<tr>
<td>Mean</td>
<td>12</td>
</tr>
<tr>
<td>SD</td>
<td>3.578</td>
</tr>
</tbody>
</table>

Comparison: Diff of Means t.test P<0.05

90000 vs 50000 8.667 3.298 0.015 S
90000 vs 70000 5 1.903 0.147 NS
70000 vs 50000 3.667 1.396 0.183 NS

SaS: Staph. aureus (sputum), StpS: S. pneumoniae (sputum), EcU: E. coli (urine), AbB: A. baumannii (blood), KpS: K. pneumoniae (sputum), PsB: P. aeruginosa (blood)
The results in tab.2 and fig.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 fig.1.

![Fig.1](image)

**Fig.1** Effect of vitamin D3 on bacterial growth (10^{10} CFU/mL )

1. StpS  
2. SaS  
3. EcU  
4. AbB  
a-Control  
b-50000  
c-70000  
d-90000  
control ; solvent only (DMSO) except for 2;control is normal saline

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

<table>
<thead>
<tr>
<th>Vitamin conc.(IU/mL)</th>
<th>50000</th>
<th>70000</th>
<th>90000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>Diameter of Inhibition Zone(mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaS</td>
<td>18</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>StpS</td>
<td>20</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>EcU</td>
<td>13</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>AbB</td>
<td>14</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>PsB</td>
<td>11</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>KpS</td>
<td>9</td>
<td>12</td>
<td>21</td>
</tr>
<tr>
<td>Mean</td>
<td>14.167</td>
<td>17</td>
<td>22.5</td>
</tr>
<tr>
<td>SD</td>
<td>4.167</td>
<td>4.243</td>
<td>5.282</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Diff of Means</th>
<th>t.test</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>90000vs50000</td>
<td>47</td>
<td>3.594</td>
<td>0.030 S</td>
</tr>
<tr>
<td>90000vs70000</td>
<td>28</td>
<td>2.141</td>
<td>0.284</td>
</tr>
<tr>
<td>70000vs50000</td>
<td>19</td>
<td>1.453</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Noticeable increases in the diameters of inhibition zone have appeared when we tested 10^{9} CFU/mL as a bacterial suspension with the different concentrations of vit.D3.Highest measurements (29mm) have recorded with SaS&StpS when treated with 90000IU/mLwhile (9.1mm) were observed for KpS,PsB respectively with 5000 IU/mL (tab.3 & fig.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 D3 has inhibitory activity on strains of S. aureus, Strep. pyogenes, K. pneumoniae, E. coli and Candida spp. in the presence of 50,000–90,000 IU/mL of vitamin D₃, 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 ).
As in tab. 4 and fig. 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.

Results shown in tab.5 were indicated coefficient 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 10⁸ CFU/mL lower than 10⁹ 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 peptid24)es . [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 D3 was lower in the intervention than in the placebo group.[26]

Inflammation resulting from the immune response targeting Propionibacterium acnes(P. acnes) has a significant role in acne pathogenesis. In a recent study, it has been demonstrated that P. acnes is a potent inducer of Th17, and that 1,25(OH)2D 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 cholonic 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 researches are needed to determine the role of vitamin D and it’s most effective bactericidal or and bacteriostatic 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.

REFERENCES


12. NCBI National Center for Biotechnology Information. USA. 2012.


