The effect of chloroquine phosphate on C-reactive protein and erythrocyte sedimentation rate measurement in knee osteoarthritic patients.

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Abstract:
Osteoarthritis (OA) is the most common articular disease world wide. It is the result of both mechanical and biological events that destabilize the normal coupling of degeneration synthesis of articular cartilage and subchondral bone.

Rheumatologist often routinely order tests for rheumatoid factor and erythrocyte sedimentation rate (ESR) for all patients with joint complaints as well as C - reactive protein (CRP) as a laboratory marker important in the
assessment of inflammation. Anti malarial drugs are used for treatment of many rheumatic diseases. Chloroquine phosphate (CQP) was used previously as a disease modifying anti rheumatic drug and in this study its effect appears through decreasing the measurement of erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in knee osteoarthritic patients (KOA).

**Abbreviation:** HCQ, hydroxy chloroquine; CQ, chloroquine; DMARD, disease modifying anti rheumatic drug; APP, acute phase protein; ACR, American College of Rheumatology; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; RF, rheumatoid factor.

**Introduction**

OA is a disease characterized by a progressive articular cartilage destruction, osteophyte formation, subchondral bone sclerosis and secondary synovitis\(^{[1,2]}\). The acute phase response is a major pathophysiologic phenomenon that accompanies acute and chronic inflammation\(^{[3,4]}\). CRP is one of APPs that influence one or more stage of inflammation so it has both pro inflammatory and anti inflammatory action\(^{[5,6]}\). ESR is an indirect measurement of plasma APPs concentration and can be greatly influenced by many factors\(^{[7]}\). Rheumatologist often routinely order tests for RF, ESR for all patients with joint complaints\(^{[8]}\). However neither the presence of RF nor mildly elevated ESR excludes a diagnosis of OA in elder patient\(^{[4]}\). CQ is an amino-quinoline derivate drug that previously used in treatment of malaria. It has a beneficial therapeutic effect in SLE, RA and viral infection\(^{[9,10,11,12]}\). Phosphate salt of CQ is used in this study to ameliorate the signs and symptoms of disease by reducing blood level of ESR and CRP.

**Materials and Methods:**

Sixty patients (40 female and 20 male) are classified as KOA by Rheumatologist according to ACR criteria\(^{[13]}\), in Out Patient Clinic in Baghdad Teaching Hospital, Medical Center, Baghdad, from January to September 2008 with fifty healthy people (30 female and 20 male). The patient ages are ranged from (55 to 67) years, their mean values ± standard mean of error are (62.7±5.2). CQP is used for one month to treat all patients, two tablets are taken daily after meal (Medoquine 250 mg /Medochem Company equivalent to 150 mg CQ base).

CRP was assessed by antigen-antibody reaction technique (quantitative turbidity metric method). ESR was estimated by Wintrob's Haematocrit tube\(^{[14]}\). Whole blood was used to determine ESR while the serum was used to determine CRP.
Results:
In this study, the presented data showed a significant (p<0.01) differences between control and patients groups before using CQP, also showed a significant (p<0.05) differences between patients group before and after treatment.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Baseline</th>
<th>P-value control-baseline</th>
<th>After one month</th>
<th>P-value pre-post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP mg/L</td>
<td>T</td>
<td>1.05±0.09</td>
<td>4.3±0.36</td>
<td>2.02±0.2</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>5.4±0.01</td>
<td>3.8±0.65</td>
<td>1.8±0.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.2±0.1</td>
<td>4.63±0.41</td>
<td>2.17±0.23</td>
<td></td>
</tr>
<tr>
<td>ESR mm/h</td>
<td>T</td>
<td>5.1±0.21</td>
<td>15.61±1.23</td>
<td>8.61±0.6</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>3.2±0.17</td>
<td>12.48±1.6</td>
<td>7.45±0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6.9±0.2</td>
<td>17.62±1.67</td>
<td>9.36±0.86</td>
<td></td>
</tr>
</tbody>
</table>

Table-1: The level of (CRP) and (ESR) before and after treatment by using (CQP) in KOA as well as control.

Were:
T = Total patient
M = Male
F = Female
The result are calculated as mean ± standard of mean, paired t-test

Figure-1: The level of serum CRP in control and KOA patients.
Discussion:

CQ is a well-known lysosomotropic agents, it can pass the plasma membrane preferentially concentrates in the acidic cytoplasmic vesicles leading to increase its PH. The elevation may influence endocytosis, exocytosis and phagocytosis [15], as well as other cell functions like antigen presentation [16], and iron metabolism [17].

CQP is present in trace concentration in the plasma of all humans. It a pentamer consisting of 5 - identical, non-covalently linked 23- KD subunit [18]. In the assessment of inflammation CRP represents an important laboratory maker as well as serves a predictor and indicator of response to therapy in addition to over all outcome in various disorder [19], so it combines phospholipids that is released from damaged tissue to become an activator of the complement pathway [20] and is useful in early detection of low-grade inflammation [21].

CQ may inhibit protein(positive APP) secretion and intracellular processing of, protein precursors such as complement precursor pro–C3 [22], decreases lymphatic proliferation and interferes with natural killer cell activity [23] and inhibits phospholipase [24]. Jawad et al previously assessed the serum level of CRP in patients with KOA at baseline and three month later of using CQP, their results showed a slight decrease (p > 0.05) in this laboratory marker [25]. The presented data in this study shows a significant decrease in CRP level (P<0.05), (Table-1), figure (1). As result, all finding, fact and trial about the
CRP serum level assessment are in agreement with this research and support it. CRP and ESR may be useful diagnostically, in helping to differentiate inflammatory from non inflammatory conditions, in patient management since they may generally reflect the response to and need for, therapeutic intervention [26].

Measurement of ESR and CRP of the patient with rheumatic disease indicates the progression and prognosis of it, as well as the elevation of both markers are associated with radiographic progression at [6,7,8,9,10,11,12] after study entry [27,28], and the time-integrated values of ESR and CRP correlate significantly with disease progression over periods of up to 20 years [29, 30], as well as their levels are significantly associated with early synovitis and erosion as detected by MRI [31] with cellular infiltrates on synovial histologic specimens [32], osteoclastic activation and reduced bone mineral density [33] and work disability on long term follow up [34].

CQ and HCQ are used previously as a DMARD, they inhibits the inflammatory response through their effects on T-cell which plays an important role in initiation and perpetuation of rheumatoid inflammation and disease progression [35, 36]. In 2004, Miranda et al studied the effect of two DMARDs combination in treatment the early onset RA, their result showed a decrease in ESR serum level after six months of using the therapy [37]. Cytokines and other inflammatory mediators are decreased because the secretion of protein is inhibited by CQ or HCQ through their lysosomotropic and non lysosomotropic action [24].

In this study the presented data showed a significant decrease in ESR measurement (p< 0.05), (Table-1), figure (2), and the result is in agreement with all findings, trials and mode of action of CQ.

Conclusion:
CQP alleviates the signs and symptoms of patients with KOA by decreasing their serum level of CRP and the blood measurement of ESR.

Further studies are needed to detect other markers and mediators in the blood and synovial fluid in relation with CQP therapy in osteoarthritic patients.

References:


Study on Dissolution test and the correlation factors that lead to different Bioavailability of Drugs

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Kassim Mahdi Wadi  
**Abdulkhaliq A. Al-Naqeeb

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**Bio-Statistician Medical & Health Technology Baghdad national center of drug control and research

Abstract:

The aim of this work is to study the physical and chemical characteristics solubility testing by using three types of drugs which are: (Clopidegrol tablet, Simvastatin tablet, Chlorpromazine tablet).
From different sources (France, Jordan, India, Germany, Creek, Syria and Lebanon) according to American standard (U.S.P30) to obtain the results to the primary solubility testing data the result of the significant level comparison to analysis the variation for the test of equal means of the data of solubility as following:

1 - The solubility of the French drug is the heights with significant level differences at:
   A - With a highly significant level (P < 0.01) Compared with the Indian product.
   B - With a non significant level at ( P > 0.05) Compared with Jordan product.
   C - A highly significant level at (P < 0.01) according to the solubility of Jordan product by using Simvastatin.

2 - The results of significant difference is based on the highest solubility by using Clopidegrol drug at (P < 0.01) compared with the Jordan and with significant level (P < 0.05) compared with Indian drug.

3 - Also the result of significant level comparison given high significant level (P < 0.01) between the German product which gives a high significant difference level compared with the Greek product and with un significant level ( P > 0.05) compared with Indian product.

4 - Show that their exist a significant difference (P < 0.05) with the highly solubility for the Lebanon product compared with Syrian product by also using chlorpromazine in similarly test for average reading of solubility by using t-test.

Introduction:
Pharmaceutical industries are subjected to an increased interest from both public groups and governments to save costs and consistently deliver to the market safe and efficient products. Therefore quality control must be able to separate kinds of products that is not suitable and at the same time acts as tool to control the production process [1].

In vitro dissolution testing serves as an important tool for characterizing the biopharmaceutical quality of a product at different stages in its lifecycle.

In early drug development in vitro dissolution properties are supportive for choosing between different alternative formulations candidates for further development and for evaluation of active ingredients/drug substances [2].

Dissolution tests are used nowadays in the pharmaceutical industry in a wide variety of applications [3].
To help identify which formulations will produce the best results in the clinic,
To release product to the market, to verify batch-to-batch reproducibility [4].
To help identify whether changes made to formulations or their manufacturing procedure after marketing [1]
Materials and Methods:
Methods:
Three drugs are selected clopidogrel, simvastatin and chlorpromazine Hcl tablets. Dissolution test was done to different company for each drug, one company of each drug are not comply in the test and the test was done with pharmacopoeia specification U.S.P.30
*for clopidogrel tablet(Hydrochloric acid buffer PH 2, paddle, 50 rpm, 30 mint, 1000 ml) limit N.L.T.80%Q, detected by U.V. at 240 nm.
*for simvastatin tablet (phosphate buffer PH 7, paddle, 50 rpm, 30 mint, 900 ml) limit N.L.T.75%Q detected by U.V. at 247 nm. And 257nm.
*for chlorpromazine Hcl tablet (0.1N Hcl, basket, 50 rpm, 30 mint, 900 ml) limit N.L.T.80%Q, detected by U.V. at 254 nm. [2]

Instruments:
Dissolution apparatus (pharma test), U.V spectrophotometer, PH meter, Ultra sound shaker and Balance.

Materials:
*Mono basic sod. Phosphate powder
*Sod. dodecyl sulphate powder
*Pot. Chloride powder
*Hcl concentrated (prep aired 0.1N Hcl)

3-primary data [6]

<table>
<thead>
<tr>
<th>No.</th>
<th>company</th>
<th>drug</th>
<th>Result of dissolution test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-</td>
<td>France</td>
<td>Clopidogrel</td>
<td>(93.2, 103, 110, 98, 101.8, 101.4)%</td>
</tr>
<tr>
<td>2-</td>
<td>Jordan</td>
<td></td>
<td>(95, 94.8, 94.5, 96.2, 95.4, 97.5)%</td>
</tr>
<tr>
<td>3-</td>
<td>India</td>
<td></td>
<td>(69.4, 36.8, 47.3, 66.3, 62.8, 81.7)%</td>
</tr>
</tbody>
</table>

Table-1: For clopidogrel tablet; Comparison in dissolution test was done for three company limit N.L.T.80%Q

<table>
<thead>
<tr>
<th>No.</th>
<th>company</th>
<th>drug</th>
<th>Result of dissolution test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-</td>
<td>Germany</td>
<td>Simvastatin</td>
<td>(84.2, 85.5, 83.8, 89.6, 90.6, 91.2)%</td>
</tr>
<tr>
<td>2-</td>
<td>Jordan</td>
<td></td>
<td>(101.8, 113, 104, 105.8, 101.5, 112)%</td>
</tr>
<tr>
<td>3-</td>
<td>India</td>
<td></td>
<td>(94.8, 95.8, 97.4, 96.8, 93.5, 90.4)%</td>
</tr>
<tr>
<td>4-</td>
<td>Greek</td>
<td></td>
<td>(46.7, 40.8, 59.5, 61.5, 37.6, 74)%</td>
</tr>
</tbody>
</table>

Table-2: For simvastatin tablet; Comparison in dissolution test was done for four company limit N.L.T.75%Q.
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<table>
<thead>
<tr>
<th>No.</th>
<th>company</th>
<th>drug</th>
<th>Result of dissolution test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-</td>
<td>Syria</td>
<td>chlorpromazine Hcl tablet</td>
<td>(97,98.5,96.7,100.2,105.9,102)%</td>
</tr>
<tr>
<td>2-</td>
<td>Lebanon</td>
<td></td>
<td>(34,40.8,55.6,50.8,47.6,42.1)%</td>
</tr>
</tbody>
</table>

Table-3: For chlorpromazine Hcl tablet; Comparison in dissolution test was done for two company limit N.L.T.80% Q

4- Descriptive statistics:

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>95% Confidence Interval for Mean</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Lower Bound</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Upper Bound</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clopidegro l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>6</td>
<td>101.23</td>
<td>5.573</td>
<td>2.275</td>
<td>95.384</td>
<td>107.082</td>
<td>93.2</td>
</tr>
<tr>
<td>Jordan</td>
<td>6</td>
<td>95.567</td>
<td>1.115</td>
<td>0.455</td>
<td>94.397</td>
<td>96.737</td>
<td>94.5</td>
</tr>
<tr>
<td>India</td>
<td>6</td>
<td>60.717</td>
<td>16.145</td>
<td>6.591</td>
<td>43.774</td>
<td>77.659</td>
<td>36.8</td>
</tr>
<tr>
<td>Simvastatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>6</td>
<td>87.483</td>
<td>3.355</td>
<td>1.37</td>
<td>83.962</td>
<td>91.004</td>
<td>83.8</td>
</tr>
<tr>
<td>Jordan</td>
<td>6</td>
<td>106.35</td>
<td>5.024</td>
<td>2.051</td>
<td>101.078</td>
<td>111.622</td>
<td>101.5</td>
</tr>
<tr>
<td>India</td>
<td>6</td>
<td>94.783</td>
<td>2.562</td>
<td>1.046</td>
<td>92.095</td>
<td>97.472</td>
<td>90.4</td>
</tr>
<tr>
<td>Greek</td>
<td>6</td>
<td>53.35</td>
<td>14.003</td>
<td>5.717</td>
<td>38.654</td>
<td>68.046</td>
<td>37.6</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>85.492</td>
<td>21.414</td>
<td>4.371</td>
<td>76.449</td>
<td>94.534</td>
<td>37.6</td>
</tr>
<tr>
<td>chlorpromazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syria</td>
<td>6</td>
<td>87.483</td>
<td>3.355</td>
<td>1.37</td>
<td>83.962</td>
<td>91.004</td>
<td>83.8</td>
</tr>
<tr>
<td>Lebanon</td>
<td>6</td>
<td>106.35</td>
<td>5.024</td>
<td>2.051</td>
<td>101.078</td>
<td>111.622</td>
<td>101.8</td>
</tr>
</tbody>
</table>

Table-4: Descriptive Statistics for Dissolution test mean values were done to different companies by using Clopidegro l &Simvastatin Drugs

<table>
<thead>
<tr>
<th>Multiple Comparisons</th>
<th>Sig.</th>
<th>C.S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA BY LSD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>France</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jordan</td>
<td>0.336</td>
<td>NS</td>
</tr>
<tr>
<td>India</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>Germany</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jordan</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>India</td>
<td>0.118</td>
<td>NS</td>
</tr>
<tr>
<td>Greek</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>Jordan</td>
<td>0.017</td>
<td>S</td>
</tr>
<tr>
<td>India</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>Greek</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>T-TEST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syria</td>
<td>0.000</td>
<td>HS</td>
</tr>
<tr>
<td>Lebanon</td>
<td>0.000</td>
<td>HS</td>
</tr>
</tbody>
</table>

Table- 5: Inferential Statistics for Dissolution test mean values were done to each pairs different companies by using Clopidegro l, Simvastatin and chlorpromazine Drugs.
Figure-1: Bar-Charts for Dissolution test mean values were done to different companies by using Clopidegrol Simvastatin and chlorpromazine Drugs

Discussion:
Dissolution is defined as the process by which a solid substance enters in solvent to yield a solution and it is controlled by the affinity between the solid substances and the solvent, the equation of no yes – wintry is described the rate of solid dissolution
\[ \frac{Dw}{Dt} = \frac{DA}{\sigma} \]

Where \( \frac{DA}{\sigma} \) is the rate of dissolution

\( Cs = \) is the solution solubility of the drug
\( C = \) is the concentration of the drug in the bulk solution
\( A = \) is the area of the solvent particles
\( \sigma = \) is thickness of the diffusion layer
\( D = \) is the diffusion coefficient of the dissolvent solute \[3\]

And there are factors which may affect dissolution rate and this different result can be contributed to it.

**Factors affecting the rate of dissolution:**

A - Factors related to the physicochemical properties of the drug.
B - Factors related to drug product formulation.
C - Effect of manufacturer process.
D - Factors related to test parameters on dissolution rate.

**Factors related to the physicochemical properties of the drug:**

A - Effect of solubility on dissolution: Aqueous solubility of the drug is the major factor that determines its dissolution rate. Some studies show that drug solubility data could be used as rough predictor of the Possibility of any future problems with bioavailability, A factor that should be taken into consideration in the Formulation design. \[4\]

B - Effect of particle size on dissolution: The dissolution rate is directly proportional to the surface area increases with the decreasing particle size, higher dissolution rate may be achieved through the reduction of the particle size. Physical properties of the drug particles other than size also affect indirectly the effective surface area by modifying the shear rate of the fresh solvent that’s come in contact with the solid; these properties include the particles shape and the density. The mechanism by which the reduction in particles size improves dissolution is usually through the enhancement of the drug solubility. \[4\]

C - Effect of solid phase characteristics of the drug on dissolution: Amorphicity and crystallinity, the two important solid-phase characteristics Of drugs affect their dissolution profile. Amorphous form of drug usually exhibits greater solubility and higher dissolution rate as compared to that exhibited by the crystalline form. For example the amorphous form of novobiocin has greater solubility and higher dissolution rate than crystalline form. Blood
level studies confirmed such findings where administration of the amorphous form yielded about three to four times the concentration compared to the administration of crystalline form. Similar differences were demonstrated for griseofulvin, Phenobarbital, Cortisone acetate and chlorramphenicol. Chlorramphenicol palmitate is one example that exists in at least two polymorphs. The polymorph B is apparently more bioavailability. The recommendation might be that Manufacturers should use polymorph B for maximum absorption.

D- Effect of polymorphism on dissolution: Numerous reports have shown that polymorphism and the state of hydration, Solvation, and/or complexation markedly influence the dissolution Characteristics of the drug. [4]

Factors related to drug product formulation:

It has shown that the dissolution rate of a pure drug can be altered significantly when mixed with various excipients during manufacturing process of solid dosage forms.

These excipients are added to satisfy certain pharmaceutical functions such as diluents (fillers), dyes, binders, granulating agent, disintegrants, and lubricants.

A- Effect of granulating agents and binders: Phenobarbital tablets granulated with gelatin solution provide faster dissolution rate in gastric fluid than those prepared using sodium carboxymethylcellulose or polyethylene glycol 6000 as a binder. This observation was attributed to the fact that gelatin imparts hydrophilic characteristics to the hydrophobic drug surface, whereas PEG 6000 forms complex with poor solubility, and sodium carboxymethylcellulose is converted to its less soluble acid from at low PH of gastric fluid.[4].

B- Effect of disintegrate and diluents: The type and amount of disintegrating agent and even the method of addition before or after the granulation all these factor effect the formulation especially the dissolution rate of the dosage form. Copagel (low viscosity grade of sodium carboxymethylcellulose) when added before granulation of Phenobarbital tablet will slow dissolution rate. However, when added after the granulation the dissolution rate will not be affected. Starch, the most commonly used diluent, the effect of increasing the content of the starch in the formulation of salicylic acid tablet lead to increasing in dissolution rate.

C- Effect of lubricants: The nature, quality and quantity of lubricants added can affect the dissolution rate. Magnesium stearate is a hydrophobic lubricant
tend to retard the dissolution rate of salicylic acid tablet, whereas sodium lauryl sulphate enhances the dissolution rate $^{[4]}$.

Factors related to the manufacturer process:
A- Method of granulation: Wet granulation has been shown to improve the dissolution rates of poorly soluble by imparting hydrophilic properties to the surface of the granules. The critical formulation and proper mixing sequence and time of adding the several ingredients are the main criteria that affect the dissolution rate. $^{[4]}$

B- Effect of compression force on dissolution rate.

Factors related to test parameters:
Eccentricity of the stirring device, guiding the shaft, vibration, Agitation intensity, Surface tension of the dissolution medium, temperature, pH of the dissolution medium. $^{[4]}$

6-interpretation:
In this study the physical and chemical characteristics solubility testing by using three types of drugs which are: (Clopidegrol tablet, Simvastatin tablet, Chlorpromazine tablet) from different sources (France, Jordan, India, Germany, Creek, Syria and Lebanon) according to American standard (U.S.P30) to obtain the results to the primary solubility testing data the result of the significant level comparison to analysis the variation for the test of equal means of the data of solubility as following:
1- The solubility of the French drug is the heights with significant level differences at:
   A - With a highly significant level (P<0.01) Compared with the Indian product.
   B - With a non significant level at (P>0.05) Compared with Jordan product.
   C - A highly significant level at (P<0.01) according to the solubility of Jordan product by using Simvastatin.
2- The results of significant difference is based on the highest solubility by using Clopidegrol drug at (P<0.01) compared with the Jordan and with significant level (P<0.05) compared with Indian drug.
3- Also the result of significant level comparison given high significant level (P<0.01) between the German product which gives a high significant difference level compared with the Greek product and with un significant level (P>0.05) compared with Indian product.
4- Showed that their exist a significant difference at (P<0.05) with the highly solubility for the Lebanon product compared with Syrian product by also
Conclusion:

Given the importance of the examination results of the test objectives give us an idea of the different pharmacological effectiveness of three types of drugs within the human body (bioavailability), especially in state companies failed for this examination. [5]

The difference in the result can be correlate to all factors which affect the dissolution rate from the raw material (purity) which can affect solubility, and all diluents which was use. [6]

Biopharmaceutical aspects are as important for stability concerns as they are for batch release after production, in vitro dissolution being of high relevance in quality control and quality assurance.

Pharmaceutical analysis today entails more than evaluation of active ingredients or formulated product, so we should understand the physic-chemical properties of drug molecules using advanced indurstructural method through studying of interactions between drug and excipients. [7]

With extensive role that had been played by analytical chemistry in development of pharmaceutical industry ,the sciences and technology utilized today have made pharmaceutical analysis more complicated compared to what it was years ago.

According to the statistical hypotheses testing , we can concludes the following results :

1 - The solubility of the French drug is the heights with significant level differences at:
   A - With a highly significant level (P<0.01) Compared with the Indian product.
   B - With a non significant level at (P>0.05) Compared with Jordan product.
   C - A highly significant level at (P<0.01) according to the solubility of Jordan product by using Simvastatin.

2 - The results of significant difference is based on the highest solubility by using Clopidegrold drug at (P<0.01) compared with the Jordan and with significant level (P<0.05) compared with Indian drug.

3 - Also the result of significant level comparison given high significant level (P<0.01) between the German product which gives a high significant difference level compared with the Greek product and with un significant level (P>0.05) compared with Indian product.

4 - Showed that their exist a significant difference at (P<0.05) with the highly solubility for the Lebanon product compared with Syrian product by also using chlorpromazine in similarly test for average reading of solubility by using t-test.
References:
2 - U.S.P. (united state pharmacopeias) 30.
New Method of Synthesis of Calix-4-arenes as Analytical Reagent for Spectrophotometric Determination of Iron (III)

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Dept. of Chemistry, College of Science, University of Karbala

Abstract:
A compound of Calix-4-arenes was prepared, as a sensitive and selective spectrophotometric method was proposed for the rapid determination of Iron (III) in analytical sample, the proposed method was based on the formation blue complex with Calix-4-arenes as a chromogenic reagent that has a highly absorption at $\lambda_{\text{max}}$ 592 nm. The reaction was instantaneous at pH=7.0, the absorbance of complex was stable for about 24hr. Linearity was observed from 1.18 -8.0 $\mu$g.ml$^{-1}$ with detection limit of 0.75$\mu$g.ml$^{-1}$. Recovery and relative error values of precision and accuracy of method were found to be R.S.D.% = 0.92%, Re% = 99.45% and Erel = 0.55 % . The nature of complex showed that (Metal : Ligand) ratio was 1:1 at pH=7 and the stability constant of (0.422x10$^{10}$ L$^{-1}$.mole$^{-1}$). The influence of chemical and physical parameter and are evaluated . The proposed method was applied successfully to determine Fe (III) in analytical sample.
**Introduction:**

The calixarenes are a class of cyclooligomers synthesized via a phenol-formaldehyde condensation. The rigid conformation calixarenes enables to act host molecules as a result of their preformed cavities. By functionally modifying either the upper and/or lower rims (the upper rim means use the bulk and long chain substitution in *para* position while the lower rim the substituent’s linkage at phenolic oxygen), it is possible to prepare various derivatives with differing selectivities for various guest ions and small molecules. Calixarenes lend themselves well to many applications because of the multiplicity of options for such structural elaboration. Calixarenes to be used in ionselective electrode (ISE) and bulk optode sensing devices, they must be immobilised into organic membranes\cite{1-3} (films/coatings) which are hydrophobic in nature. Furthermore, if aqueous analysis is desired, the calixarenes must be water insoluble otherwise immobilisation would be undermined by water dissolution of the ionophore from the device. It is essential that the calixarenes used in this way be lipophilic with very low water soluble materials.\cite{4-8}

The McKervey group began work on calixarene synthesis back in the 1980s. Their first major accomplishment in the field involved the modification of the lower rims of the tetra-, hexa- and octa- calixarenes by the introduction of a series of acetate esters. The calixarene ester derivatives were shown to have characteristics which make them attractive agents for use in potentiometric ion sensors. The resulting calixarenes demonstrated outstanding selectivity for various cations. Calixarenes displayed selectivity toward the sodium cation. It was reasoned that the presence of the t-butyl groups in the upper rim forced the calixarene into a permanent cone conformation which allowed the esters to form the necessary cavity for coordination\cite{8-10}.

In this work, synthesis, characterization and analytical study of Calix-4-arenes where used as organic reagent for spectrophotometric determination of Fe(III), also a new spectrophotometric method is described for the determination of Fe(III) in analytical sample which that use in the work.

**Materials and Methods:**

**Instruments**

Infrared spectrum was recorded on FT-IR Test scan Shimadzu model 8400 Fourier transform infrared spectrophotometer covering the rang 400-4000 cm\(^{-1}\). The electronic spectrum was recorded in ethanol on Unico model 4802UV/Vis double beam spectrophotometer recording. \(^1\)H-NMR spectrum in DMSO d\(^6\) with TMS as internal standard were obtained from a JeolFX-90Q Fourier \(^1\)H-NMR spectrometer at university of London ,college of Queen Marry. Molar conductance of solid
complex in DMSO (dimethyl sulfoxide) was measured by using WTW-Terminal 740 digital conductivity meter. pH measurements were carried out using WTW pH meter model 720. Gallenkamp capillary melting point apparatus was used to measure the melting points of the ligand and its complexes.

**Reagents:**
All chemicals were used in the present investigation are of analytical garde from Fluka and Aldrich companies
All solutions were prepared with distilled water.

**Preparation of [2,6-dimethylol 4–methyl phenol]**

To a solution of NaOH (50g, 125mmole) in H$_2$O (200mL) was added $p$-cresol (108g, 100mmole). The mixture was stirred until a clear brown solution was obtained, stirring was continued while a (37%) solution of (CH$_2$O) (formaldehyde) (215g, 200mmole) in H$_2$O was added with contentious stirring. The yellow solution was allowed to stand for 48hrs., after which time a white solid had precipitated. This was filtered and washed with saturated NaCl solution (200mL). The white precipitate was dried under vaccum. The Na salt [Na(2,6-dimethylol-4–methyl phenolate] was dissolved in 1.5 times the amount of water and neutralized with dilute acetic acid with stirring. The free alcohol crystallized out as white crystals, recrystallised from acetone– water (3:1) ratio, filtered and dried to give 130g, (90%) product, melting point (128°C).\(^{[11]}\)

**Preparation of [Calix]-4- arenes**

The condensation reaction of four equivalent of 2,6–dimethylol 4–methyl phenol (100 mmole, 67.2 g) in (200 mL) of O-xylene , the mixture was stirred half hour, then allowed to reflux to four hours, the orange precipitate was formed, the result precipitate washed in (5 mL) cold methanol, and (25 mL) 2% citric acid , the two layer solution were obtained, separated by equalized funnel, the organic layer take and dried by MgSO$_4$, filtered , the filtrate dried by vacuum, the deep yellow precipitate yielded, 65g, (33%). The preparation of ligand was showed in Scheme1.

![Scheme-1: Synthesis route of Calix-4-arenes ligand](image.png)
Preparation the solid complex of Fe(III)

(0.2 mmole) ligand mixed with 15mL of ethanol, then 0.2mmole of FeCl$_3$·6H$_2$O that dissolved with a minimum amount of ethanol (5 mL) in a 50ml beaker, that precipitate by reflex about 2hr. the complex filtrated and purification, than dried and stored in tightly closed container. Some physical properties of the prepared ligand and it's complex were tabulated in (Table-1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight(g/mole)</th>
<th>$\lambda_{\text{max}}$ nm</th>
<th>Color</th>
<th>Melting point</th>
<th>Conductivity S.mol$^{-1}$.cm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td>480.590</td>
<td>306</td>
<td>Yellow-brown</td>
<td>108 C°</td>
<td>6.42</td>
</tr>
<tr>
<td>Fe (III)-complex</td>
<td>601.432</td>
<td>592</td>
<td>blue</td>
<td>230 C° Dec.</td>
<td>369.4</td>
</tr>
</tbody>
</table>

Table-1: Some physical properties of reagent and Fe (III)-complex

Results and Discussion
I-Characterization of ligand
Electronic spectra

The ultra violet spectrum of bis( hydroxy methyl) p-cresol and calyx-4-arenş Fig (1) and Fig (2) respectively, appeared , peaks at 284nm ($\varepsilon_{\text{max}} = 1154$ molar$^{-1}$ cm$^{-1}$) of starting material and (245,284 nm) ($\varepsilon_{\text{max}} = 1383,1135$ molar$^{-1}$ cm$^{-1}$)due to the $\pi \rightarrow \pi^*$ and n→$\pi^*$ respectively

Fig-1: UV-Vis spectrum of 2,6–dimethylol 4–methyl phenol
Fig-2: UV-Vis spectrum of [calix]-4-arenes

\(^1\)H NMR spectra

The \(^1\)H NMR spectrum of [calix]-4- arenes Fig (3) shows signal at (\(\delta=8.35\) ppm ,4H) assigned to Ar-OH groups , The signals at (\(\delta=7.10\) ppm ,4H , \(\delta=6.25\)ppm ,4H ) attributed to aromatic protons of four benzene rings, due to the aromatic rings are exist in twested configuration the Ar-CH\(_2\) groups appears twin signals at (\(\delta=3.50,3.20\) ppm ,8H ) and methy groups Ar-CH\(_3\) shows twin signal at (\(\delta=2.25,2.10\)ppm ,12 H ),the signal at (\(\delta=2.60\) ppm ) assigned the solvent DMSO-d\(_6\).

Fig-3: \(^1\)H NMR spectrum of [calix] -4- arenes
FT-IR spectra

The IR spectrum of *bis* (hydroxyl methyl)-p-cresal (Fig-4) displayed two bands at 3400 cm\(^{-1}\) and 3308 cm\(^{-1}\) can be aromatic and aliphatic hydroxyl groups respectively. The weak bands at 3050cm\(^{-1}\) and (2893)cm\(^{-1}\) assigned to ν(C-H) stretching of aromatic aliphatic (C-H) respectively , the other bands at (1568,1442 and 1205 )cm\(^{-1}\) due to the vibration frequencies of rings . While the IR spectra of (calyx)-4-arenes . (Fig-5) shows the disappearing of band at 3400 cm\(^{-1}\) due to aliphatic (OH) and the shifting of other bands indicating of the obtaining of calix-4-areanes.

![Fig-4: FT-IR spectrum of 2,6-dimethylol 4-methyl phenol](image)

![Fig-5: FT-IR spectrum of [calix]-4-arenes](image)
II-Characterization of complex

Electronic spectra

The electronic spectral data of the ligand and its complexes were recorded in DMSO. The UV-Visible spectrum of the ligand, Fig (2) shows twin absorption peak at (245, 284 nm) \( (\varepsilon_{\text{max}} = 1383,1135 \text{ molar}^{-1} \text{cm}^{-1}) \) due to the \( \pi \rightarrow \pi^* \) and due \( n \rightarrow \pi^* \) transitions respectively\[^{12}\]. While the electronic spectrum of the Fe(III) complex Fig (6) exhibited the following data.

The Fe(III) complex shows peak at 252 nm \( (39682 \text{cm}^{-1}) \) \( (\varepsilon_{\text{max}} = 3700 \text{ molar}^{-1} \text{cm}^{-1}) \) and at 335 nm \( (30030 \text{cm}^{-1}) \) \( (\varepsilon_{\text{max}} = 3920 \text{ molar}^{-1} \text{cm}^{-1}) \) assigned to intraligand charge transfer, the peak at \( (592 \text{nm}(16891) \text{ cm}^{-1}) \) \( (\varepsilon_{\text{max}} = 651 \text{ molar}^{-1} \text{cm}^{-1}) \) and shoulder peak at \( 960 \text{nm},(10416 \text{ cm}^{-1}) \) \( (\varepsilon_{\text{max}} = 230 \text{ molar}^{-1} \text{cm}^{-1}) \) that attributed to d-d transition, which are suggesting the existence of \( ^4T_1g \rightarrow ^4T_2g \) and \( ^4T_1g \rightarrow ^4A_1g \) transitions which that corresponding with an octahedral geometry of Fe(III) ion[^{13}]

![Fig-6: UV-Vis spectrum of Fe(III)-complex](image)

IR spectra

The infrared band of the ligand and complex of Fe(III) assignments that the broad band at \( 3400 \text{cm}^{-1} \) in the free ligand spectrum Fig(5) which assigned to \( \nu(-\text{OH}) \) stretching[^{14}], the lower frequency for \( \nu(-\text{OH}) \) due to hydrogen bonding[^{15}]. This band in IR spectrum of complex was shifted and reduced intensity due to complex formation(2) There is also a shifting noticed in the \( \nu \) (C-O) of phenol group from \( 1232 \text{cm}^{-1} \) toward \( 1390 \text{cm}^{-1} \) producing another evidence about involvement of phenol group in coordination with metal ions \( \text{via} \) oxygen atom[^{16}].

New bands in the region \( 451 \text{cm}^{-1} \) were assigned in the spectra of metal complexes. These bands were not present in the spectrum of ligand, and they due to
(M-O) vibration\textsuperscript{[17]}. The appearance of these bonds support the involvement of hydroxyl groups and oxygen atoms in complexation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{FT-IR spectrum of Fe(III)-complex}
\end{figure}

\textbf{Optimization study of the Fe (III)-complex}

Fe (III) ion react with Calix-4-arenes in neutral medium to form a violet complex that has a maximum absorption at $\lambda_{max}$ 592 nm Figr (6). The absorbance of the blue complex is directly related to the concentration of Fe (III) and that can be used for its spectrophotometric determination. The development of the color intensity and stability of complex depends on the reaction condition and were optimize as follows:

\textbf{Influence of pH}

The pH was studied between (3-9) adjusted by means of dilute HCl and NaOH solution, where the maximum absorbance obtained in the range of (pH =7). At pH > 7 a decreases in absorbance because the precipitation of Fe(III) complex or form unstable ionic complexes\textsuperscript{[18]}.

\textbf{Influence of time:}

The color intensity reached a maximum after Fe(III) solution had been reacted immediately with reagent and become stable after 10 min, therefore 10 min. development time was selected as optimum in the general procedure. The blue complex was stable for 24 hr.

\textbf{Order addition effect}

The effect of order addition in absorbance of formation complex, depended a two sequence that tabulated in \textit{Table 2}
Table-2: Effect of order addition, \([\text{Fe}^{3+}] = 10 \, \mu\text{g.mL}^{-1}, [\text{L}] = 200 \, \mu\text{g.mL}^{-1}\)

<table>
<thead>
<tr>
<th>No. of test</th>
<th>Sequence of addition</th>
<th>Abs. of Fe(III)-complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M+L+pH</td>
<td>0.092</td>
</tr>
<tr>
<td>2</td>
<td>L+M+PH</td>
<td>0.117</td>
</tr>
</tbody>
</table>

**Influence of metal concentration**

When various concentration of metal solution in the range of (1–60 \(\mu\text{g.mL}^{-1}\)) were added to fixed amount of reagent [Calix]-4- arenes 200 \(\mu\text{g.mL}^{-1}\). Concentrations of metal 40 \(\mu\text{g.mL}^{-1}\) give the highest absorbance Fig (8) and were for further experiments.

**Influence of reagent concentration**

When various concentration of [Calix]-4- arenes solution in the range of (5 – 250 \(\mu\text{g.mL}^{-1}\)) were added to fixed amount of Fe(III) 40 \(\mu\text{g.mL}^{-1}\). Concentrations of reagent 200 \(\mu\text{g.mL}^{-1}\) give the highest absorbance Fig (9) and were for further experiments.
Stoichiometry of complex

The stoichiometry of the complex was investigated using mole – ratio and job method under the optimizes conditions. The result obtained Fig (10) and Fig (11), show a 1:1 metal to reagent complex was formed. The formation of the complex may probable be occur as follows

\[ \text{Job's method of Fe (III)-complex} \]

As applied to the molar ratio method, stability constants are obtained spectrophotometricaly by measuring the absorbance of solutions of ligand and metal mixture at fixed wavelength \( \lambda \text{max} \) and pH values. The degree of formation of the complexes is obtained according to the relationship \[ [19], K = (1 - \alpha) / \alpha^2 c, \] and \[ \alpha = (Am - As)/Am, \] where As and Am are the absorbance's of the partially and fully formed complex respectively at optimum concentration. The complex have stability constant 0.415 \( x10^{10} \ L.mole^{-1} \) that appear the complex highly stability which can used a reagent to spectrometric determination of Fe(III).
Calibration curve

Under the optimum condition linear calibration graph Fig (12), was obtained over the concentration range of (1.18 - 8.00 µg.ml⁻¹). The limited of detection (signal/ noise = 3) was -0.75 µg.ml⁻¹ and the correlation coefficient was 0.974.

![Calibration curve of Fe (III)-complex](image)

**Precision and Accuracy**

The relation standard deviation evaluated from five independent determination of 10 µg.ml⁻¹ of Fe(III) was 0.92%, this result show that the method is highly precise while the accuracy of the method was determined by calculating the Erel% and Re% for and 10 µg.ml⁻¹ of Fe (III) which was found to be 0.55% and 99.45% respectively.

**Influence of foreign ions**

The selectivity of the proposed methods was investigated by the determination (10 µg.ml⁻¹) of Fe (III) in the presence of a series of 10 µg.ml⁻¹ of cations and 100 µg.ml⁻¹ anions at pH =7 (Table 3 and Table 4 respectively). The results show the tolerance limit was taken as the amount that caused an error of 0.112 in the absorbance.
Table-3: Determination tolerance limits of some foreign cations

<table>
<thead>
<tr>
<th>Species</th>
<th>Absorbance of Fe(III) complex</th>
<th>Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex without addition</td>
<td>0.112</td>
<td>---</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>0.072</td>
<td>35.71</td>
</tr>
<tr>
<td>Zr$^{4+}$</td>
<td>0.103</td>
<td>8.04</td>
</tr>
<tr>
<td>Al$^{3+}$</td>
<td>0.115</td>
<td>-2.68</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>0.089</td>
<td>20.53</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>0.096</td>
<td>14.29</td>
</tr>
<tr>
<td>Cd$^{2+}$</td>
<td>0.103</td>
<td>8.036</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.108</td>
<td>3.57</td>
</tr>
</tbody>
</table>

Table-4: Determination tolerance limits of some foreign anions

<table>
<thead>
<tr>
<th>Species</th>
<th>Absorbance of Fe(III) complex</th>
<th>Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex without addition</td>
<td>0.112</td>
<td>---</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>0.096</td>
<td>14.29</td>
</tr>
<tr>
<td>F$^-$</td>
<td>0.001</td>
<td>99.11</td>
</tr>
<tr>
<td>C$_2$O$_4^{2-}$</td>
<td>0.077</td>
<td>31.25</td>
</tr>
<tr>
<td>Ac$^-$</td>
<td>0.034</td>
<td>69.64</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>0.085</td>
<td>24.11</td>
</tr>
<tr>
<td>CrO$_4^{2-}$</td>
<td>0.098</td>
<td>12.50</td>
</tr>
<tr>
<td>SCN$^-$</td>
<td>0.063</td>
<td>43.75</td>
</tr>
</tbody>
</table>

Which they are also reacting with Calix-4-arenes reagent at the same choosing conditions and they were masked by using suitable masking agent. The results obtained are summarized in Table 5.
Table-5: Effect of masking agents

<table>
<thead>
<tr>
<th>Masking agent 0.5 ml (0.01M)</th>
<th>Absorbance of Fe(III)-Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex without addition</td>
<td>0.112</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.010</td>
</tr>
<tr>
<td>Thiourea</td>
<td>0.017</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.012</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Table-6: Application data of determination of Fe (III) in clay sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration of Fe(III) prepared µg/ml</th>
<th>Absorbance measured</th>
<th>Concentration of Fe(III) measured µg/ml</th>
<th>wt/wt% measured of Fe(III)</th>
<th>Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepared Fe(III)</td>
<td>10</td>
<td>0.134</td>
<td>9.945</td>
<td>99.45%</td>
<td>0.55%</td>
</tr>
<tr>
<td>Clay sample (Pantonat)</td>
<td>------</td>
<td>0.180</td>
<td>13.34</td>
<td>2.7%</td>
<td>------</td>
</tr>
</tbody>
</table>

Suggestion of structural formula of Fe(III) complex

According to these results from FT-IR spectrum, absorption spectra study and conductivity measurement while the composition of the complex was studied by Job's method and mole ratio method. Both methods indicated that the ratio of metal ion to ligand molecules was 1:1(M:L). The octahedral geometry around Fe(III) ion can be suggested, as follow.
References:
4- Naranchimeg Dorjpalam; Masaya Toda; Hideakiltoh and Fumio Hamada (2004). Fluorescent Molecular Sensing System Based on Tri-Pyrenes-Labeled γ-Cyclodextrin at the Hetero Rim, *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 50, 79–85,


Studying the effect of silymarin against oxidative stress induced by chemotherapeutic protocol in breast cancer women


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Abstract

Breast cancer became the commonest type of cancers among Iraqi women since the last two decades. The main underlying cause is thought to be DNA damage; much of which is oxidative in nature. CAF protocol (Cyclophosphamide + Adriamycin + 5-FU) associated with toxic effects in several body organs, mainly through production of free radicals and reactive oxygen species. Silymarin, the dried extract of a ripe seeds of the plant *silybum marianum*, was found to be a powerful antioxidant protective agent against toxin -induced tissue damage .The objective of this study is to evaluate the possible time and dose-dependent protective effect of the orally administered silymarin as antioxidant agent against oxidative stress induced by CAF protocol (mainly Adriamycin) in breast cancer women. This study included 94 subjects, 20 were healthy control women (for matching with oxidative stress markers) and 74 were breast cancer women that randomly distributed and allocated into three groups:

Group (A): Include 24 patients who received CAF protocol by I.V infusion once every 21 days and for 63 days.

Group (B): Include 25 patients who received 210mg/day of along with the same CAF protocol of group (A);

Group (C): Include 25 patients who received 420mg/day of silymarin along with the same CAF protocol of group (A). Oxidative stress markers (MDA and GSH) were measured at baseline (zero time), after 21, 42, and 63 days of treatment for each patient group.

Our results showed an increase in the oxidative stress for both baseline patients and those treated with CAF protocol, manifested by significant increase in...
MDA levels and GSH depletion, a state which is significantly reversed by use of silymarin, in a time and dose-dependent manner. Breast cancer and its antineoplastic CAF protocol produce free radicals which attenuate antioxidant defense mechanism of the body leading to several toxic effects on different body organs, so the use of antioxidant agent (silymarin) in this study may ameliorate, in a time and dose-dependant manner, the harmful effects of this protocol.

**Keywords:** Breast cancer, CAF protocol (cyclophosphamide+adriamycin+5-fluorouracil) Oxidative stress, silymarin.

**Introduction:**
Breast cancer is a malignant tumor that has developed from cells of the breast. It occurs almost in women, but men can get it, too [1]. According to cancer registry section (Iraqi Cancer Board) Baghdad / Ministry of Health, breast carcinoma is the most common malignant tumor in Iraqi women and it comprise (31.3%) of all female malignant cases. [2]. There are different kinds of risk factors. Some factors, like a person's age or race, can’t be changed .Others are linked to cancer-causing factors in the environment. Still others are related to personal choices such as smoking, drinking, and diet [3].

Breast cancer usually begin in the cells that line the ducts (ductal cancer), some begin in the cells that line the lobules (lobular cancer), and the rest in the other tissues. Breast cancer classified according to WHO [4] into: non-invasive (non-infiltrating) or in situ carcinoma; and invasive (infiltrating) carcinoma. The most beneficial and commonly used staging system of breast cancer is the American Joint Committee on Cancer (AJCC) classification, which is based on the tumor size (T), the status of regional lymph nodes (N), and the presence of distant metastasis (M) [5]. There are three major techniques that are commonly used to evaluate breast masses, excluding surgical procedures. These are: physical examination, mammography, and fine needle aspiration cytology (FNAC) [6].

Local treatment (surgery or radiation) and systemic treatment (hormonal or chemotherapy) can be planned by number of ways. The most common sequence is: surgery - chemotherapy - radiation – and then hormonal therapy. Combination of two or three chemotherapeutic drugs is used in breast cancer to avoid drug resistance and for better response. Several such combination regimens or protocols are available, such as CAF (Cyclophosphamide + Adriamycin +5-FU), and CMF (Cyclophosphamide + Methotrexate+5-FU) [7].

Adriamycin, or Doxorubicin, is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius var. caesius*; Cyclophosphamide is a synthetic antineoplastic drug chemically related to the nitrogen mustards; while 5-Fluorouracil (5-FU) is a pyrimidine analog (5-fluoro-2, 4(1H,3H)-pyrimidinedione).The use of these cytotoxic drugs against breast cancer is limited
Silymarin is a mixture of flavonolignans isolated from the ripe seeds of the medicinal plant *Silybum marianum* (milk thistle), comprised mainly of silybinin (SBN) (A,B) isosilybinin (ISBN), silychristin (SCN), silydianin (SDN) and taxifolin (TXF)\cite{9}. Multiple biological effects of flavonoids have been described, including anti-inflammatory, anti-allergic, anti-haemorrhagic, anti-mutagenic, anti-neoplastic, and hepatoprotective activities \cite{10}. Most flavonoids, including silibinin, can protect cells and tissues against the harmful effects of reactive oxygen species (ROS). Their antioxidant activity results from scavenging of free radicals and other oxidizing intermediates, chelation of iron or copper ions and inhibition of oxidases \cite{11}. Flavonoids from *Silybum marianum* have been widely used for the treatment of liver disorders. In experimental animal models, they exerted not only a positive effect on intact liver cells or cells not yet irreversibly damaged, but also to stimulate their regenerative capacity after partial hepatectomy \cite{12}. No adverse reactions have been reported due to silymarin use in rats or human; either with short term or in long-lasting therapy \cite{13}.

The aim of the present study was to evaluate the possible time and dose-dependent effects of the orally-administered silymarin as a protective agent against oxidative stress which could be induced by CAF protocol in women with breast cancer.

**Materials, Subjects and Methods:**

Chemicals, drugs, and instruments that were used in this study are mentioned with their manufactures and origins in (tables-1,2,3) respectively. This randomized clinical study was carried out on 80 female patients with different stages of breast cancer, all pass through one type of operative mastectomy and this is the first time they receive chemotherapy in their life’s. These patients were with age range of 41-60 years (mean: 49± 1.5) and body weight range of 65-96 kg (mean: 76± 2.5). Certain exclusion criteria were followed to avoid interference of any other factors and include: those with history of previous chemotherapy, cardiac disorders, pregnant and breast feeding women, and those for whom any of CAF protocol components is contraindicated.
### Table-1: Chemicals used in the study

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacture</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,5-dithio- bis (2-nitrobenzoic acid): DTNB</td>
<td>BDH chemicals, Ltd.</td>
<td>Poole, England</td>
</tr>
<tr>
<td>Thiobarbituric acid : TBA</td>
<td>BDH chemicals, Ltd.</td>
<td>Poole, England</td>
</tr>
<tr>
<td>Trichloroacetic acid: TCA</td>
<td>Merck chemical</td>
<td>Germany</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
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<tr>
<td>Potassium dihydrogen phosphate (KH₂PO₄)</td>
<td>Merk, chemicals</td>
<td>Germany</td>
</tr>
</tbody>
</table>

### Table-2: Drugs used in the study

<table>
<thead>
<tr>
<th>Drug</th>
<th>Manufacture</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>Ebewe Phrma</td>
<td>Austria</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Baxter</td>
<td>Germany</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>Ebewe Phrma</td>
<td>Austria</td>
</tr>
<tr>
<td>Silymarin Standardized Powder</td>
<td>Luna Company</td>
<td>Egypt</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Instrument</th>
<th>Manufacture</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold Centrifuge -Universal 16A</td>
<td>Hettich</td>
<td>Germany</td>
</tr>
<tr>
<td>UV Spectrophotometer</td>
<td>Jenway 6300</td>
<td>U.K</td>
</tr>
<tr>
<td>pH meter pw 9420</td>
<td>Philips</td>
<td>Netherlan ds</td>
</tr>
<tr>
<td>Water Bath WB22</td>
<td>Memmert Lab.</td>
<td>Germany</td>
</tr>
<tr>
<td>Autovortex SA60</td>
<td>Stuart Scientific</td>
<td>U.K</td>
</tr>
<tr>
<td>Freezer</td>
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<td>Germany</td>
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</table>
Only 74 female patients completed the study, others were excluded due to poor compliance with the follow up program. These patients were diagnosed and treated in Baghdad Teaching Hospital/ Department of Surgery/ Unit of Oncology under follow up of specialist doctors during the period from March 2009 to September 2009. Our patients were randomly allocated in three groups as follow:

**Group (A):** Include 24 patients who received CAF protocol (Cyclophosphamide 600 mg/m² + Adriamycin 60 mg/m²+ 5- FU 600 mg/m²) by intravenous infusion once every 21 days and for 63 days.

**Group (B):** Include 25 patients who received 210mg/day of silymarin (given as single dose in a capsule dosage form especially prepared for this purpose) along with same CAF protocol of group (A).

**Group (C):** Include 25 patients who received 420mg/day of silymarin (given as 210mg/12hour in a capsule dosage form especially prepared for this purpose) along with same CAF protocol of group (A).

Twenty healthy females were involved and considered as control group to compare the results of their oxidative stress markers with that of patient groups.

After over night fasting, venous blood (5 ml) was obtained from the forearm of each patient by vein puncture at a baseline before the initiation of therapy, after 21, 42 days of treatment and at the end of 63 days for all patient groups. Each blood sample was placed in EDTA-free tube to be centrifuged for 10 minutes at 3000rpm. Serum was then divided into several eppendorf tubes and kept frozen until time for the assay.

Malondialdehyde (MDA), the end product of lipid peroxidation, was analyzed according to the method of Buege and Aust [14] based on the reaction of MDA with thiobarbituric acid (TBA) to form a red chromophore, which can be quantitated spectrophotometrically. Total thiol group contents, which can be used as a marker for the reduced glutathione (GSH), were determined according to the method of Ellman [15], where 0.5 ml of serum was added to 4.5 ml of 5,5 – dithiobis -(2nitro benzoic acid) DTNB reagent [0.1 mM DTNB in 0.1 M phosphate buffer pH =8]. The light absorbence of the solution at 412nm was measured after 2 minutes.

**Statistical analysis:**

The results were expressed as mean ± standard error of mean (SEM). Student’s paired t-test and ANOVA test were used to examine the degree of significance and P values < 0.05 were considered significant.

**Results:**

At baseline, breast cancer produced significant elevation (P<0.05)in serum MDA levels (146%,158%,164%) for patients treated with CAF protocol ,CAF protocol and 210 or 420 mg/day of silymarin, respectively compared to control
Further significant elevation ($P<0.05$) in serum MDA levels was observed as a result of treatment with CAF protocol (43%, 80%, 94%) after 21, 42 and 63 days, respectively compared with baseline. Significant reduction ($P<0.05$) in serum MDA levels was observed for patients treated with CAF protocol and 210 mg/day of silymarin (21%, 41%, 58%) and those treated with CAF protocol and 420 mg/day of silymarin (23%, 43%, 60%) after 21, 42, and 63 days of treatment, respectively compared with baseline values. There was significant difference ($P<0.05$) in serum MDA levels for patients treated with CAF protocol and silymarin (210 or 420 mg/day) after the end of each treatment cycle compared with those received just CAF protocol. At the end of 63 days of treatment with CAF protocol and silymarin (210 or 420 mg/day), serum MDA levels were comparable ($P>0.05$) to that of control values (table 4).

At baseline, breast cancer produced significant reduction ($P<0.05$) in serum GSH levels (40%, 42%, 41%) for patients treated with CAF protocol, CAF protocol and 210 or 420 mg/day of silymarin, respectively compared to control subjects (table 5). Further significant reduction ($P<0.05$) in serum GSH levels was observed as a result of treatment with CAF protocol (15%, 31%, 48%) after 21, 42 and 63 days, respectively compared with baseline. Significant elevation ($P<0.05$) in serum GSH levels was observed for patients treated with CAF protocol and 210 mg/day of silymarin (15%, 32%, 50%) and those treated with CAF protocol and 420 mg/day of silymarin (15%, 33%, 68%) after 21, 42, and 63 days of treatment, respectively compared with baseline values. There was significant difference ($P<0.05$) in serum GSH levels for patients treated with CAF protocol and silymarin (210 or 420 mg/day) after the end of each treatment cycle compared with those received just CAF protocol. Meanwhile, the elevation in this parameter values was significant ($P<0.05$) after 63 days of treatment with CAF protocol and 420 mg/day of silymarin (14%) compared with those on CAF protocol and 210 mg/day of silymarin. At the end of 63 days of treatment with CAF protocol and 420 mg/day of silymarin, serum GSH levels were comparable ($P>0.05$) to control values (table 5).
### Table-4: Effects of treatment with 210 and 420 mg/day of silymarin on serum MDA levels in breast cancer patients treated with CAF protocol.

Results were expressed as mean± SEM
Results with non identical superscripts (a, b, c,d) within the same group were considered significantly different at \(P<0.05\)
† = Significant at \(P<0.05\) as compared with CAF protocol values
\(\Psi\) = Significant at \(P<0.05\) as compared with control values

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Serum MDA (µmol/l)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>21 days post</td>
<td>42 days</td>
<td>63 days</td>
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<tr>
<td></td>
<td></td>
<td>treatment</td>
<td>post</td>
<td>post</td>
<td>post</td>
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<tr>
<td>Control</td>
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<td>0.998±0.01</td>
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<tr>
<td>CAF protocol</td>
<td>24</td>
<td>2.45±0.47 (^{a,\Psi})</td>
<td>3.51±0.12 (^b)</td>
<td>4.42±0.32 (^c)</td>
<td>4.75±0.27 (^c)</td>
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<tr>
<td>CAF+Sily. (210mg/day)</td>
<td>25</td>
<td>2.57±0.23 (^{a,\Psi})</td>
<td>2.03±0.11 (^{b\Psi})</td>
<td>1.52±0.09 (^{c\Psi})</td>
<td>1.07±0.23 (^{d\Psi})</td>
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<tr>
<td>CAF+Sily. (420mg/day)</td>
<td>25</td>
<td>2.63±0.37 (^{a,\Psi})</td>
<td>2.02±0.08 (^{b\Psi})</td>
<td>1.49±0.13 (^{c\Psi})</td>
<td>1.04±0.17 (^{d\Psi})</td>
</tr>
</tbody>
</table>

### Table-5: Effects of treatment with 210 and 420 mg/day of silymarin on serum GSH levels in breast cancer patients treated with CAF protocol.

Results were expressed as mean± SEM
Results with non identical superscripts (a, b, c,d) within the same group were considered significantly different at \(P<0.05\)
† = Significant at \(P<0.05\) as compared with CAF protocol values
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<table>
<thead>
<tr>
<th>Group</th>
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<tbody>
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<td>post</td>
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<tr>
<td>Control</td>
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<tr>
<td>CAF protocol</td>
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<td>0.192±0.02 (^{a,\Psi})</td>
<td>0.163±0.04 (^b)</td>
<td>0.132±0.07 (^c)</td>
<td>0.1±0.03 (^d)</td>
</tr>
<tr>
<td>CAF+Sily. (210mg/day)</td>
<td>25</td>
<td>0.185±0.14 (^{a,\Psi})</td>
<td>0.213±0.15 (^{b\Psi})</td>
<td>0.244±0.12 (^{c\Psi})</td>
<td>0.277±0.18 (^{d\Psi})</td>
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<tr>
<td>CAF+Sily. (420mg/day)</td>
<td>25</td>
<td>0.188±0.12 (^{a,\Psi})</td>
<td>0.217±0.21 (^{b\Psi})</td>
<td>0.250±0.1 (^{c\Psi})</td>
<td>0.315±0.16 (^{d\Psi})</td>
</tr>
</tbody>
</table>
Discussion:

Regarding oxidative stress and breast cancer relationship, the results presented in this study clarified that, in breast cancer patients and at baseline, serum MDA levels were significantly higher, while serum GSH levels were significantly lower, than that of control, which may be attributed to the overproduction of ROS, a state of systemic oxidative stress, and deficiency of antioxidant defense mechanism (table 4,5). These results support the oxidative stress hypothesis in carcinogenesis which was studied by Kumaraguruparan et al. and Khanzode et al., who found an increase in lipid peroxidation in plasma and solid tumors \[^{16,17}\].

Our results were consistent with those reported by Huang et al. and Gonenç et al., who found significant increase in plasma MDA levels in cancer patients compared with normal subjects \[^{18,19}\]. These results were also consistent with that recorded by Ray et al., who studied lipid peroxidation, free radical production, and antioxidant status in breast cancer patients. The rate of superoxide (O2·⁻) and hydrogen peroxide (H2O2) production was significantly higher in breast cancer patients than normal \[^{20}\].

Concerning the effect of CAF protocol on oxidative stress, formation of free radicals via electron reduction is the mechanism supposed to induce cytotoxicity by doxorubicin \[^{21}\]. Doxorubicin is quinine which can undergo one-electron reduction to semiquinone or two-electron reduction to the corresponding dihydroquinone derivatives. The semiquinone reacts rapidly with oxygen to generate O2·⁻, which can be dismutated to H2O2, which in turn reacts in various ways to produce hydroxyl radicals. Hydroxyl radicals can react with polyunsaturated fatty acids, initiating a lipid-radical chain reaction and oxidative damage to cell membrane. Increased levels of ROS due to metabolism of doxorubicin have been detected by an increase in tissue MDA levels \[^{22}\]. In the presence of transition metal ions, the chain reaction continues and free iron appears to play a particularly important role in doxorubicin- inducing lipid peroxidation. Without free iron, MDA formation is minimal and even a low concentration of free iron can lead to substantial MDA production \[^{23}\]. Doxorubicin may act by transferring an electron directly to Fe³⁺ and the produced Fe²⁺ can reduce oxygen to hydrogen peroxide. Redox cycling of doxorubicin in this manner also generates free radical metabolites and ROS \[^{24}\].

The above hypothesis was supported by our study, in which there was significant elevation in serum MDA levels and significant reduction in serum GSH levels for patients who received doxorubicin (within CAF protocol) along treatment duration compared with their baseline values (table 4,5). These results were consistent with that reported by Eser Özl and Mustafa, who found that, in doxorubicin-treated animals, the MDA levels of kidney, lung, liver, and brain tissues were significantly increased, compared to control rats \[^{25}\]. Veselina et al.
reported that plasma GSH levels were significantly reduced in lymphoproliferative cancer patients treated with doxorubicin [26].

In addition to doxorubicin, cyclophosphamide (within CAF protocol) can also considered as a source of ROS and contribute in the elevation of MDA levels and GSH depletion. Both cyclophosphamide and ifosfamide are metabolized to acrolein, which is responsible for the stimulation of oxidative stress, and then bladder toxicity. The co-administration of MESNA (sodium 2-mercaptoethane sulfonate), a sulphydryl-containing compound which binds to acrolein, has reduced the incidence of haemorrhagic cystitis associated with ifosfamide and high dose of cyclophosphamide [27].

Regarding the effect of silymarin on CAF-induced oxidative stress, silymarin antioxidant activity was studied by Rastogi et al on aflatoxin B1-induced lipid peroxidation in rat liver and kidney, where treatment with silymarin reversed the increase in lipid peroxide levels and increased the antioxidant levels near the normal values [28]. The antioxidant effect of silymarin was observed in rats intoxicated by acetaminophen and ethanol, which are an oxidant stress inducers that produce marked MDA elevation and GSH depletion in the liver. Treatment with silymarin or silybinin was able to protect animals against oxidative stress produced in the liver by these chemicals [29]. Another study performed by Valenzuela showed that, when liver from rats pre-treated in vivo with intravenous silybinin (50 mg/kg), there was significant reduction in the oxygen consumption and MDA release stimulated by phenyl hydrazine, without any changes in GSH levels [30].

Data reported in this study support the antioxidant effect of silymarin, where the administration of silymarin with CAF protocol significantly decrease serum MDA levels and increase serum GSH levels along the study compared with their baseline values, in time and dose-dependent manner. After the end of treatment course, the levels of MDA and GSH were comparable to control values (table 4,5). Silymarin antioxidant activity can not be related only to the reduction of MDA production and GSH elevation. It has been reported that this effect included also improvement in the activities of many antioxidant enzymes including glutathione reductase (GSH-R), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) [31].

References:
AJPS, 2011, Vol. 10, No.2

J.; Lawrence, R. et al. (Eds); Cancer Management: A Multidisciplinary approach. 9th Ed., 175-202.
AJPS, 2011, Vol. 10, No.2


Studying the effect of silymarin against oxidative stress induced by chemotherapeutic protocol in breast cancer women


College of Pharmacy, Al-Must. Univ.*, **College of Medicine, Baghdad Univ.
AJPS, 2011, Vol. 10, No.2

Breast cancer became the commonest type of cancers among Iraqi women since the last two decades. The main underlying cause is thought to be DNA damage; much of which is oxidative in nature. CAF protocol (Cyclophosphamide + Adriamycin + 5-FU) associated with toxic effects in several body organs, mainly through production of free radicals and reactive oxygen species. Silymarin, the dried extract of a ripe seeds of the plant *silybum marianum*, was found to be a powerful antioxidant protective agent against toxin-induced tissue damage. The objective of this study is to evaluate the possible time and dose-dependent protective effect of the orally administered silymarin as antioxidant agent against oxidative stress induced by CAF protocol (mainly Adriamycin) in breast cancer women. This study included 94 subjects, 20 were healthy control women (for matching with oxidative stress markers) and 74 were breast cancer women that randomly distributed and allocated into three groups:

Group (A): Include 24 patients who received CAF protocol by I.V infusion once every 21 days and for 63 days.

Group (B): Include 25 patients who received 210mg/day of along with the same CAF protocol of group (A);

Group (C): Include 25 patients who received 420mg/day of silymarin along with the same CAF protocol of group (A). Oxidative stress markers (MDA and GSH) were measured at baseline (zero time), after 21, 42, and 63 days of treatment for each patient group.

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Abstract

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Our results showed an increase in the oxidative stress for both baseline patients and those treated with CAF protocol, manifested by significant increase in
MDA levels and GSH depletion, a state which is significantly reversed by use of silymarin, in a time and dose-dependent manner. Breast cancer and its antineoplastic CAF protocol produce free radicals which attenuate antioxidant defense mechanism of the body leading to several toxic effects on different body organs, so the use of antioxidant agent (silymarin) in this study may ameliorate, in a time and dose-dependent manner, the harmful effects of this protocol.

**Keywords:** Breast cancer, CAF protocol (cyclophosphamide+adriamycin+5-fluorouracil) Oxidative stress, silymarin.

**Introduction:**
Breast cancer is a malignant tumor that has developed from cells of the breast. It occurs almost in women, but men can get it, too [1]. According to cancer registry section (Iraqi Cancer Board) Baghdad / Ministry of Health, breast carcinoma is the most common malignant tumor in Iraqi women and it comprise (31.3%) of all female malignant cases [2]. There are different kinds of risk factors. Some factors, like a person's age or race, can’t be changed. Others are linked to cancer-causing factors in the environment. Still others are related to personal choices such as smoking, drinking, and diet [3].

Breast cancer usually begin in the cells that line the ducts (ductal cancer), some begin in the cells that line the lobules (lobular cancer), and the rest in the other tissues. Breast cancer classified according to WHO [4] into: non-invasive (non-infiltrating) or in situ carcinoma; and invasive (infiltrating) carcinoma. The most beneficial and commonly used staging system of breast cancer is the American Joint Committee on Cancer (AJCC) classification, which is based on the tumor size (T), the status of regional lymph nodes (N), and the presence of distant metastasis (M) [5]. There are three major techniques that are commonly used to evaluate breast masses, excluding surgical procedures. These are: physical examination, mammography, and fine needle aspiration cytology (FNAC) [6].

Local treatment (surgery or radiation) and systemic treatment (hormonal or chemotherapy) can be planned by number of ways. The most common sequence is: surgery - chemotherapy - radiation –and then hormonal therapy. Combination of two or three chemotherapeutic drugs is used in breast cancer to avoid drug resistance and for better response. Several such combination regimens or protocols are available, such as CAF (Cyclophosphamide + Adriamycin +5-FU), and CMF (Cyclophosphamide + Methotrexate+5-FU) [7].

Adriamycin, or Doxorubicin, is a cytotoxic anthracycline antibiotic isolated from cultures of *Streptomyces peucetius var. caesius*; Cyclophosphamide is a synthetic antineoplastic drug chemically related to the nitrogen mustards; while 5-Fluorouracil (5-FU) is a pyrimidine analog (5-fluoro-2, 4(1H,3H)-pyrimidinedione). The use of these cytotoxic drugs against breast cancer is limited
by number of adverse effects and toxicities, including cardiotoxicity, nephrotoxicity, hepatotoxicity, neurotoxicity, myelosuppression and blood disorders. Much of these complications attributed to the induction of oxidative stress by CAF protocol (especially by doxorubicin)\textsuperscript{[8]}.

Silymarin is a mixture of flavonolignans isolated from the ripe seeds of the medicinal plant \textit{Silybum marianum} (milk thistle), comprised mainly of silybinin (SBN) (A,B) isosylbilibinin (ISBN), silychristin (SCN), silydianin (SDN) and taxifolin (TXF)\textsuperscript{[9]}. Multiple biological effects of flavonoids have been described, including anti-inflammatory, anti-allergic, anti-haemorrhagic, anti-mutagenic, anti-neoplastic, and hepatoprotective activities\textsuperscript{[10]}. Most flavonoids, including silibinin, can protect cells and tissues against the harmful effects of reactive oxygen species (ROS). Their antioxidant activity results from scavenging of free radicals and other oxidizing intermediates, chelation of iron or copper ions and inhibition of oxidases\textsuperscript{[11]}. Flavonoids from \textit{Silybum marianum} have been widely used for the treatment of liver disorders. In experimental animal models, they exerted not only a positive effect on intact liver cells or cells not yet irreversibly damaged, but also to stimulate their regenerative capacity after partial hepatectomy\textsuperscript{[12]}. No adverse reactions have been reported due to silymarin use in rats or human; either with short term or in long-lasting therapy\textsuperscript{[13]}

The aim of the present study was to evaluate the possible time and dose-dependent effects of the orally-administered silymarin as a protective agent against oxidative stress which could be induced by CAF protocol in women with breast cancer.

Materials, Subjects and Methods:

Chemicals, drugs, and instruments that were used in this study are mentioned with their manufactures and origins in (tables-1,2,3) respectively. This randomized clinical study was carried out on 80 female patients with different stages of breast cancer, all pass through one type of operative mastectomy and this is the first time they receive chemotherapy in their life’s. These patients were with age range of 41-60 years (mean: 49$\pm$ 1.5) and body weight range of 65-96 kg (mean: 76$\pm$ 2.5). Certain exclusion criteria were followed to avoid interference of any other factors and include: those with history of previous chemotherapy, cardiac disorders, pregnant and breast feeding women, and those for whom any of CAF protocol components is contraindicated.
### Table-1: Chemicals used in the study

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacture</th>
<th>Origin</th>
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<tr>
<td>Thiobarbituric acid : TBA</td>
<td>BDH chemicals, Ltd.</td>
<td>Poole, England</td>
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<tr>
<td>Trichloroacetic acid: TCA</td>
<td>Merck chemical</td>
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<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
<td>Fluka-Garantie</td>
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Twenty healthy females were involved and considered as control group to compare the results of their oxidative stress markers with that of patient groups.

After over night fasting, venous blood (5 ml) was obtained from the forearm of each patient by veine puncture at a baseline before the initiation of therapy, after 21, 42 days of treatment and at the end of 63 days for all patient groups. Each blood sample was placed in EDTA-free tube to be centrifuged for 10 minutes at 3000rpm.Serum was then divided into several eppendorf tubes and kept frozen until time for the assay.

Malondialdehyde (MDA), the end product of lipid peroxidation, was analyzed according to the method of Buege and Aust [14] based on the reaction of MDA with thiobarbituric acid (TBA) to form a red chromophore, which can be quantititated spectrophotometrically. Total thiol group contents, which can be used as a marker for the reduced glutathione (GSH), were determined according to the method of Ellman [15], where 0.5 ml of serum was added to 4.5 ml of 5,5 – dithiobis -(2nitro benzoic acid) DTNB reagent [0.1 mM DTNB in 0.1 M phosphate buffer pH =8]. The light absorbence of the solution at 412nm was measured after 2 minutes.

**Statistical analysis:**

The results were expressed as mean ± standard error of mean (SEM). Student’s paired t-test and ANOVA test were used to examine the degree of significance and P values < 0.05 were considered significant.

**Results:**

At baseline, breast cancer produced significant elevation (P<0.05)in serum MDA levels (146%,158%,164%) for patients treated with CAF protocol ,CAF protocol and 210 or 420 mg/day of silymarin, respectively compared to control
Further significant elevation ($P<0.05$) in serum MDA levels was observed as a result of treatment with CAF protocol (43%, 80%, 94%) after 21, 42 and 63 days, respectively compared with baseline. Significant reduction ($P<0.05$) in serum MDA levels was observed for patients treated with CAF protocol and 210 mg/day of silymarin (21%, 41%, 58%) and those treated with CAF protocol and 420 mg/day of silymarin (23%, 43%, 60%) after 21, 42, and 63 days of treatment, respectively compared with baseline values. There was significant difference ($P<0.05$) in serum MDA levels for patients treated with CAF protocol and silymarin (210 or 420 mg/day) after the end of each treatment cycle compared with those received just CAF protocol. At the end of 63 days of treatment with CAF protocol and silymarin (210 or 420 mg/day), serum MDA levels were comparable ($P>0.05$) to that of control values (table 4).

At baseline, breast cancer produced significant reduction ($P<0.05$) in serum GSH levels (40%, 42%, 41%) for patients treated with CAF protocol, CAF protocol and 210 or 420 mg/day of silymarin, respectively compared to control subjects (table 5). Further significant reduction ($P<0.05$) in serum GSH levels was observed as a result of treatment with CAF protocol (15%, 31%, 48%) after 21, 42 and 63 days, respectively compared with baseline. Significant elevation ($P<0.05$) in serum GSH levels was observed for patients treated with CAF protocol and 210 mg/day of silymarin (15%, 32%, 50%) and those treated with CAF protocol and 420 mg/day of silymarin (15%, 33%, 68%) after 21, 42, and 63 days of treatment, respectively compared with baseline values. There was significant difference ($P<0.05$) in serum GSH levels for patients treated with CAF protocol and silymarin (210 or 420 mg/day) after the end of each treatment cycle compared with those received just CAF protocol. Meanwhile, the elevation in this parameter values was significant ($P<0.05$) after 63 days of treatment with CAF protocol and 420 mg/day of silymarin (14%) compared with those on CAF protocol and 210 mg/day of silymarin. At the end of 63 days of treatment with CAF protocol and 420 mg/day of silymarin, serum GSH levels were comparable ($P>0.05$) to control values (table 5).
Table 4: Effects of treatment with 210 and 420 mg/day of silymarin on serum MDA levels in breast cancer patients treated with CAF protocol.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Serum MDA (µmol/l)</th>
<th>Baseline</th>
<th>21 days post treatment</th>
<th>42 days post treatment</th>
<th>63 days post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0.998±0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF protocol</td>
<td>24</td>
<td>2.45±0.47 a,Ψ</td>
<td>3.51±0.12 b</td>
<td></td>
<td>4.42±0.32 c</td>
<td>4.75±0.37 c</td>
</tr>
<tr>
<td>CAF+Sily. (210mg/day)</td>
<td>25</td>
<td>2.57±0.23 a,Ψ</td>
<td>2.03±0.11 b†</td>
<td></td>
<td>1.52±0.09 c†</td>
<td>1.07±0.23 d†</td>
</tr>
<tr>
<td>CAF+Sily. (420mg/day)</td>
<td>25</td>
<td>2.63±0.37 a,Ψ</td>
<td>2.02±0.08 b†</td>
<td></td>
<td>1.49±0.13 c†</td>
<td>1.04±0.17 d†</td>
</tr>
</tbody>
</table>

Table 5: Effects of treatment with 210 and 420 mg/day of silymarin on serum GSH levels in breast cancer patients treated with CAF protocol.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Serum GSH (µmol/l)</th>
<th>Baseline</th>
<th>21 days post treatment</th>
<th>42 days post treatment</th>
<th>63 days post treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0.321±0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAF protocol</td>
<td>24</td>
<td>0.192±0.02 a,Ψ</td>
<td>0.163±0.04 b</td>
<td></td>
<td>0.132±0.07 c</td>
<td>0.1±0.03 d</td>
</tr>
<tr>
<td>CAF+Sily. (210mg/day)</td>
<td>25</td>
<td>0.185±0.14 a,Ψ</td>
<td>0.213±0.15 b†</td>
<td></td>
<td>0.244±0.12 c†</td>
<td>0.277±0.18 d†</td>
</tr>
<tr>
<td>CAF+Sily. (420mg/day)</td>
<td>25</td>
<td>0.188±0.12 a,Ψ</td>
<td>0.217±0.21 b†</td>
<td></td>
<td>0.250±0.1 c†</td>
<td>0.315±0.16 d†</td>
</tr>
</tbody>
</table>
Discussion:

Regarding oxidative stress and breast cancer relationship, the results presented in this study clarified that, in breast cancer patients and at baseline, serum MDA levels were significantly higher, while serum GSH levels were significantly lower, than that of control, which may be attributed to the overproduction of ROS, a state of systemic oxidative stress, and deficiency of antioxidant defense mechanism (table 4,5). These results support the oxidative stress hypothesis in carcinogenesis which was studied by Kumaraguruparan et al. and Khanzode et al., who found an increase in lipid peroxidation in plasma and solid tumors [16,17].

Our results were consistent with those reported by Huang et al. and Gönenç et al., who found significant increase in plasma MDA levels in cancer patients compared with normal subjects [18,19]. These results were also consistent with that recorded by Ray et al., who studied lipid peroxidation, free radical production, and antioxidant status in breast cancer patients. The rate of superoxide (O2·-) and hydrogen peroxide (H2O2) production was significantly higher in breast cancer patients than normal [20].

Concerning the effect of CAF protocol on oxidative stress, formation of free radicals via electron reduction is the mechanism supposed to induce cytotoxicity by doxorubicin [21]. Doxorubicin is quinine which can undergo one-electron reduction to semiquinone or two-electron reduction to the corresponding dihydroquinone derivatives. The semiquinone reacts rapidly with oxygen to generate O2·-, which can be dismutated to H2O2, which in turn reacts in various ways to produce hydroxyl radicals. Hydroxyl radicals can react with polyunsaturated fatty acids, initiating a lipid-radical chain reaction and oxidative damage to cell membrane. Increased levels of ROS due to metabolism of doxorubicin have been detected by an increase in tissue MDA levels [22]. In the presence of transition metal ions, the chain reaction continues and free iron appears to play a particularly important role in doxorubicin-inducing lipid peroxidation. Without free iron, MDA formation is minimal and even a low concentration of free iron can lead to substantial MDA production [23]. Doxorubicin may act by transferring an electron directly to Fe2+ and the produced Fe2+ can reduce oxygen to hydrogen peroxide. Redox cycling of doxorubicin in this manner also generates free radical metabolites and ROS [24].

The above hypothesis was supported by our study, in which there was significant elevation in serum MDA levels and significant reduction in serum GSH levels for patients who received doxorubicin (within CAF protocol) along treatment duration compared with their baseline values (table 4,5). These results were consistent with that reported by Eser Özl and Mustafa, who found that, in doxorubicin-treated animals, the MDA levels of kidney, lung, liver, and brain tissues were significantly increased, compared to control rats [25]. Veselina et al.
reported that plasma GSH levels were significantly reduced in lymphoproliferative cancer patients treated with doxorubicin \cite{26}.

In addition to doxorubicin, cyclophosphamide (within CAF protocol) can also considered as a source of ROS and contribute in the elevation of MDA levels and GSH depletion. Both cyclophosphamide and ifosfamide are metabolized to acrolein, which is responsible for the stimulation of oxidative stress, and then bladder toxicity. The co-administration of MESNA (sodium 2-mercaptoethane sulfonate), a sulphhydryl-containing compound which binds to acrolein, has reduced the incidence of haemorrhagic cystitis associated with ifosfamide and high dose of cyclophosphamide \cite{27}.

Regarding the effect of silymarin on CAF-induced oxidative stress, silymarin antioxidant activity was studied by Rastogi et al. on aflatoxin B1-induced lipid peroxidation in rat liver and kidney, where treatment with silymarin reversed the increase in lipid peroxide levels and increased the antioxidant levels near the normal values \cite{28}. The antioxidant effect of silymarin was observed in rats intoxicated by acetaminophen and ethanol, which are an oxidant stress inducers that produce marked MDA elevation and GSH depletion in the liver. Treatment with silymarin or silybinin was able to protect animals against oxidative stress produced in the liver by these chemicals \cite{29}. Another study performed by Valenzuela showed that, when liver from rats pre-treated in vivo with intravenous silybinin (50 mg/kg), there was significant reduction in the oxygen consumption and MDA release stimulated by phenyl hydrazine, without any changes in GSH levels \cite{30}.

Data reported in this study support the antioxidant effect of silymarin, where the administration of silymarin with CAF protocol significantly decrease serum MDA levels and increase serum GSH levels along the study compared with their baseline values, in time and dose-dependent manner. After the end of treatment course, the levels of MDA and GSH were comparable to control values (table 4,5). Silymarin antioxidant activity can not be related only to the reduction of MDA production and GSH elevation. It has been reported that this effect included also improvement in the activities of many antioxidant enzymes including glutathione reductase (GSH-R), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) \cite{31}.

References:
AJPS, 2011, Vol. 10, No.2

J.; Lawrence, R. et al. (Eds); Cancer Management: A Multidisciplinary approach. 9th Ed., 175-202.


The Antibacterial Activity of Callemia Sinensis (green tea) on Some Bacterial Species.

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And Hana B. Dalya
College of Pharmacy, University of Mustansiriya.

Abstract:

The main objective of this research is to screen and to evaluate the antibacterial activity of methanol extract of Callemia Sinensis and to find out zone of inhibition against gram positive (G+ve) bacteria as well as gram negative (G-ve) bacteria.

Extraction of the leaves Callemia Sinensis (green tea) and tested against 54 isolates of G+ve and G-ve bacteria which were isolated from different samples, which include pus of skin, urine and throat swabs of patients who were admitted to Al-jerahat specialized hospital in Baghdad during the period of 1/12/2008 to 1/7/2009.

The methanolic extract of the plant Callemia Sinensis (green tea) showed antibacterial activity against 54 bacterial isolates that would be isolated in this study: 8 isolates of Escherichia coli, 8 isolates of Streptococcus pneumoniae, 12 isolates of Klebsiella pneumoniae, 8 isolates of Staphylococcus aureus, 6 isolates of Proteus species, and 12 isolates of Pseudomonas aeruginosa.
Callemia Sinensis have been shown to have potential chemotherapeutic activities. These data provide a direct mechanism of action for Callemia Sinensis and further support its role as a chemopreventive agent. 

**Key words:** Callemia Sinensis, antibacterial activity, G+ve bacteria, G-ve bacteria.

**Introduction:**

Callemia Sinensis (green tea) is rich in the polyphenolic compounds-bounded benzene rings with multiple hydroxyl groups, and non flavonoid. Tea polyphenols are strong antioxidant, improve gastrointestinal function and alcohol metabolism, it improve, kidney, liver and pancreatic function, protect skin and eyes, and alleviate arthritis.[1]

Tea has been used in managing and preventing allergies, diabetes, viral infection, tooth cavities to reduce acute disease with an inflammatory component and to improve psychological health [2,3].

Hexane a chemical component of Callemia Sinensis (green tea) and many flavonoids including antibacterial activities of tea extracts and catchiness [4,5].

The main objective of this research to screen and evaluate antibacterial activity of methanol extract of Callemia Sinensis and to find out zone of inhibition against both G+ve and G-ve bacteria.

**Materials and Methods:**

**Plant Materials:**

The leaves of Callemia Sinensis were purchased from local market in Baghdad city.

The leaves of Callemia Sinensis (green tea) were cut into pieces, 25 gm of the leaves were soxhlet extracted using 350 milliliters of 95% methanol. The extraction tested for ten hours. The crude obtained was concentrated by evaporation using rotary evaporator at 100°C [6].

**Microorganism:**

Test organisms were collected from Al-jerahat specialized hospital in Baghdad during the period of from 1/12/2008 to 1/7/2009: Escherichia coli, Pseudomonas aeruginos and Proteus species were isolated from urine samples of patients with urinary tract infections. Streptococcus pneumoniae were isolated from throat swabs of patients with tonsillitis. Klebsiella
*pneumoniae* were isolated from sputum of patients with upper respiratory tract infections. *Staphylococcus aureus* were isolated from patients with skin abscesses.

**Determination of zone of inhibition:**

Fifteen millimeters (15 ml) of sterile nutrient agar was poured into each sterile Petri dish of equal size and allowed to solidify. The surface of this sterile nutrient agar plate was streaked with pure culture of the standardized bacterial cell suspension. Acrokborer (8 millimeter in diameter) was sterilized by flaming and used to create ditch at the center of the plate. The hole so created was then filled with the (50%) concentration of plant extract. The plates were allowed to stand for one hour for pre-diffusion of the extract [7] and incubation was done at 37 °C for 24 hours. At the end of incubation period, the diameter of zone of inhibition was measured in millimeters [8].

**Results:**

The activity was evaluated using the agar diffusion method by measuring the diameter of the growth inhibition. Methanol extracts of *Callemia Sinensis* (green tea) plant used into traditional medicine of Iraq were submitted to a screening for antibacterial activity against six strains (table-1). 

(Table-2) show the extracts obtained from *Callemia Sinensis* were found to be effective against *Escherichia coli* used in this study in different level.

(Table-3) show the extracts obtained from *Callemia Sinensis* were found to be effective against *Streptococcus pneumoniae*.

(Table-4) shows the methanolic extracts obtained from *Callemia Sinensis* were found to be effective against *Klebsiella pneumoniae*.

(Table-5) shows the methanolic extract obtained from *Callemia Sinensis* were found to be effective against *Staphylococcus aureus*.

(Table-6) show the methanolic extracts obtained from *Callemia Sinensis* were found to be effective against *Proteus* species.

(Table-7) show the methanolic extracts obtained from *Callemia Sinensis* were found to be effective against *Pseudomonas aeruginosa*. 
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<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>No. of positive strains for antibacterial effect of <em>Callemia Sinensis</em> (green tea)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td><em>Str. Pneumoniae</em></td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td><em>Kleb. Pneumoniae</em></td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td><em>S. aureus</em></td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td><em>Proteus spp</em></td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td><em>Ps. aeruginosa</em></td>
<td>12</td>
</tr>
</tbody>
</table>

Table-1: Number of each bacterial species inhibited by antibacterial effect of *Callemia Sinensis* (green tea).

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Ten fold dilutions of <em>Callemia Sinensis</em> extract</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻¹  10⁻²  10⁻³  10⁻⁴  10⁻⁵  10⁻⁶  10⁻⁷  10⁻⁸  10⁻⁹  10⁻¹⁰ Cipr o</td>
</tr>
<tr>
<td>1</td>
<td>12    12    10    8    7    6    4    3    2    1    20</td>
</tr>
<tr>
<td>2</td>
<td>10    9     8     7     5     4     3     2     1     1    20</td>
</tr>
<tr>
<td>3</td>
<td>12    11    8     -ve  5     3     2     -ve  1     1    20</td>
</tr>
<tr>
<td>4</td>
<td>11    10    8     6     5     3     2     2     1     -ve  20</td>
</tr>
<tr>
<td>5</td>
<td>10    9     8     7     6     4     3     2     1     -ve  20</td>
</tr>
<tr>
<td>6</td>
<td>12    11    10    8     7     5     3     3     2     -ve  20</td>
</tr>
<tr>
<td>7</td>
<td>10    9     7     6     5     4     3     -ve  2     1     20</td>
</tr>
<tr>
<td>8</td>
<td>11    9     8     8     7     5     4     3     2     1     20</td>
</tr>
</tbody>
</table>

Table 2: Zone of inhibition diameters of the ten fold dilutions of methanolic extract of *Callemia Sinensis* on the *E. coli* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Dilutions(ten fold dilutions) of extract of <em>Callemia Sinensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Str.pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻¹  10⁻²  10⁻³  10⁻⁴  10⁻⁵  10⁻⁶  10⁻⁷  10⁻⁸  10⁻⁹  10⁻¹⁰ Cipr o</td>
</tr>
<tr>
<td>1</td>
<td>11    11    10    9     7     6     5     2     1     1     27</td>
</tr>
<tr>
<td>2</td>
<td>15    14    12    10    9     8     5     4     3     2     27</td>
</tr>
<tr>
<td>3</td>
<td>12    10    10    9     7     6     4     3     2     1     27</td>
</tr>
<tr>
<td>4</td>
<td>12    11    9     7     6     5     4     2     1     1     27</td>
</tr>
<tr>
<td>5</td>
<td>13    11    8     8     7     -ve  5     3     2     1     27</td>
</tr>
<tr>
<td>6</td>
<td>12    11    10    9     8     7     6     5     4     2     27</td>
</tr>
<tr>
<td>7</td>
<td>13    12    9     8     7     7     -ve  5     3     2     27</td>
</tr>
<tr>
<td>8</td>
<td>12    10    8     6     5     4     3     2     1     1     27</td>
</tr>
</tbody>
</table>

Table-3: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *Str.pneumoniae* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).
Table-4: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *Kleb.pneumoniae* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Ten fold dilutions of <em>Callemia Sinensis</em> extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻¹</td>
</tr>
<tr>
<td><em>Kleb. pneumoniae</em></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>22</td>
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<tr>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>9</td>
<td>14</td>
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<tr>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>16</td>
</tr>
</tbody>
</table>

Table-5: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *S.aureus* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Dilutions of <em>Callemia Sinensis</em> extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻¹</td>
</tr>
<tr>
<td><em>S.aureus</em></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
</tr>
</tbody>
</table>
Table-6: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *Proteus spp* isolates compared with antibacterial effects of ciprofloxacin disc (5 µg).

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Ten fold dilutions of <em>Callemia Sinensis</em> extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Proteus spp</em></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>

Table-7: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *Ps.aeruginosa* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Dilutions(ten fold dilutions) of green tea extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Ps.aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
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<td>6</td>
<td>10</td>
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<tr>
<td>7</td>
<td>11</td>
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<td>10</td>
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<tr>
<td>10</td>
<td>17</td>
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<tr>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
</tr>
</tbody>
</table>

Table-6: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *Proteus spp* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).

Table-7: Diameter of zone of inhibition (mm) of the methanol extract of *Callemia Sinensis* on the *Ps.aeruginosa* isolates compared with antibacterial effects of ciprofloxacin disc (5µg).

Discussion:

Green tea extends the effectiveness of sun screen and has been shown to have strong anti-irritant, anti-inflammatory and antibacterial properties for cleaner and healthier looking skin [9]. However, *Callemia Sinensis* have been to have potential chemo preventive activities. The extracts obtained from *Callemia Sinensis* (green...
tea) were found to be effective against all tested microorganism used in this study at different level, showing several studies\textsuperscript{[10,11,12,13]} have been conducted on the antibacterial properties of herbs spices and their derivatives such as essential oils, extracts and decoctions. In vitro studies in this work showed that the plant extracts inhibited bacterial growth but their effectiveness varied. This result agreed with Hamilton and Otuke\textsuperscript{[14,15]} who suggested that the antibacterial activity of \textit{Callemia Sinensis} was properly due to their major component. The inhibition produced by the plant extracts against particular organism depends upon various extrinsic and intrinsic parameters. Due to variable diffusability in agar medium, the antibacterial property may not demonstrate as zone inhibition commensurate to its efficacy.

All extracts were active against \textit{E.coli}, \textit{Streptococcus pneumoniae}, \textit{Klebsiella pneumoniae}, \textit{Staphylococcus aureus}, \textit{Proteus} species and \textit{Pseudomonas aeruginosa}.

This result not agreed with Firas,\textsuperscript{[16]} who reported that \textit{Callemia Sinensis} had antibacterial activities against all pathogenic bacteria except \textit{Proteus mirabilis}.

Ping et al 2008\textsuperscript{[17]} reported treatment with these agents may represent important adjuncts to, or alternative to, conventional antibiotic therapy.

Further evaluation of the antibacterial properties of these extracts and elucidation of the components responsible for the activities is warranted.

\textbf{Conclusions}: \textit{Callemia Sinensis} have been shown to have potential chemotherapeutic activities. These data provide a direct mechanism of action for \textit{Callemia Sinensis} and further support of its role as a chemopreventive agents.

\textbf{References}


Formulation and In-Vitro Evaluation of Mucoadhesive Diltiazem Hydrochloride Buccal Tablets

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Abstract

Mucoadhesive tablets for buccal administration of diltiazem hydrochloride were prepared as an alternative to available diltiazem HCl dosage forms. Two types of tablets were developed each containing two mucoadhesive components (hydroxypropylmethyl cellulose HPMC and sodium alginate) and (HPMC and carbopol) for each types, batches were produced by changing quantity of polymer. The formulations were tested for mucoadhesive performance and release pattern. In vitro bioadhesive strength studies showed that the HPMC/carbopol formulations were more bioadhesive and less drug release rate compared with HPMC/alginate formulations. Increasing the content of HPMC in HPMC/alginate tablets resulted in increase in detachment forces and swelling index but lower release rates were observed. The release behavior of all formulations was non-Fickian mechanism controlled by a combination of diffusion and chain relaxation mechanisms and best fitted zero-order kinetics. The buccoadhesive diltiazem HCl tablets containing 18.75% sodium alginate
and 37.5% HPMC showed suitable release kinetics (n = 0.86, K₀ zero order release = 10.29 mg/h, MDT = 4.8 h), good adhesive properties and did not show any interaction between polymers and drug based on FT-IR study.

**Introduction:**

Among the various routes of drug delivery, oral route is perhaps the most preferred to the patient. However, peroral administration of drugs has disadvantages such as hepatic first pass metabolism and enzymatic degradation within the GI tract, that prohibit oral administration of certain classes of drugs especially peptides and proteins. Drug buccal administration, on the other hand, has many advantages such as rich vascularity, moderate permeability, suitability for both local and systemic drug delivery, less enzymatic activity and avoidance of first pass metabolism [1]. The accessibility of buccal cavity makes the application of drugs easy and acceptable to patient, while permitting easy removal in the event of adverse reaction [2]. Also reduced costs of the drug because of application of much lower doses than necessary for oral products.

The major limitation associated with buccal route of administration is the lack of dosage form retention at the site of absorption. Consequently, during the past decade, bioadhesive polymers have received considerable attention for platforms of buccal controlled delivery because of their ability to localize the dosage form in specific regions to enhance drug bioavailability [3].

Therefore, bioadhesive polymers have extensively been employed and adhesive mucosal dosage forms are suggested for buccal delivery, including adhesive tablets [4], adhesive gels [5], adhesive film and patches [6, 7].

Diltiazem HCl is a calcium channel blocker widely used for its peripheral and vasodilator properties. It is also used for lowering blood pressure and has some effect on cardiac induction. It is given as oral dosage form in the treatment of angina pectoris and the management of hypertension. It has short biological half life (3.5 h) and subjected to extensive first pass effect. The oral bioavailability of diltiazem HCl is 40% in humans [8] make it a suitable candidate for buccal controlled release preparations.

The aim of this study is development and characterization of a buccoadhesive controlled-release tablet of diltiazem HCl using some hydrophilic polymers like carbopol 940 (CP), hydroxypropylmethyl cellulose (HPMC), and sodium alginate (SA). bioadhesions and in vitro release characteristics of diltiazem HCl from different buccoadhesive matrix tablets was evaluated to assess the suitability of such formulations.
Materials and Methods:

Diltiazem HCl (United Pharmaceutical, Jorden), carbopol 940 (J.Baker, USA), hydroxypropylmethyl cellulose 2280 (metolose 90sh 4000 SR, Seppic, Japan), sodium alginate (Himedia Lab, Mumbia, India), polyvinylpyrrolidone K-30 (Samar Drug Industry), All other reagents and chemicals used were of analytical reagent grade.

Formulation of mucoadhesive tablets:

Mucoadhesive tablets were prepared by direct compression method using the formula shown in Table 1. The drug and other excipients was mixed homogenously in glass mortar and then lubricated with 1% magnesium stearate. Finally, compressed into tablets using single punch tablet machine (Manesty Type F3, England).

Evaluation of physical properties of mucoadhesive tablets:

The thickness, hardness and friability were determined in a similar manner as stated for conventional oral tablets. Friability was determined by subjecting 20 tablets to falling shocks in friablator (Roche friablator, England) for 4 min at 25 rpm. Hardness of the tablets was determined using Monsanto hardness tester [9].

Drug content uniformity:

Five tablets from each formulation were crushed and each tablet was weighed. then extracted with 20 ml of phosphate buffer pH 6.4 and was centrifuged at 4000 rpm for 10 min, the supernatant was then analyzed after dilution with buffer in such a way that theoretical concentration was same as that of standard concentration. Resultant solutions were analyzed by using a spectrophotometer (Carry UV, Varian, Australia) at 237 nm [10].

Surface pH study:

The designed tablets were first allowed to swell in contact with 5mL of distilled water (pH 6.5 ± 0.05) for 2 h. The surface pH was measured by bringing glass electrode of pH meter (Hanna Instrument pH 221 Microprocessor, Italy) in contact with the surface of tablets and allowing it to equilibrate for 1 min. The surface pH of the tablets was determined in order to investigate the possibility of any discomfort in oral cavity as acidic or alkaline pH may lead to irritation [3].

Swelling studies:

Buccal tablets were weighed individually (W1) and placed separately in 2% agar gel surface in Petri dish and incubated at 37 ± 1°C. At regular 1-hour time intervals until 6 hours, the tablet was removed from the Petri dish and excess surface water was removed carefully using filter paper. The swollen tablet was then reweighed (W2) and the swelling index (SI) were calculated using the following formula [11].

\[
SI = \frac{(W_2 - W_1)}{W_1} \times 100
\]
Ex vivo mucoadhesion time:
The ex vivo mucoadhesion time was examined after application of the buccal tablet on freshly cut sheep buccal mucosa. The fresh sheep buccal mucosa was tied on the glass slide, and a mucoadhesive tablet was wetted with 1 drop of phosphate buffer pH 6.8 and pasted to the sheep buccal mucosa by applying a light force with a fingertip for 30 seconds. The glass slide was then put in the beaker, which was filled with 200 ml of the phosphate buffer pH 6.8 and kept at 37°C ± 1°C. After 2 minutes, a slow stirring rate was applied to simulate the buccal cavity environment, and tablet adhesion was monitored for 12 hours. The time for detachment or complete erosion of tablets from the sheep buccal mucosa was recorded as the mucoadhesion time [3].

Ex vivo mucoadhesive strength:
Bioadhesive strength of the tablets was measured by using a modified balance method described by Emami [12]. Briefly, fresh sheep buccal mucosa (2x2cm) was tied to the open mouth of smaller beaker which was filled completely with phosphate buffer pH 6.8 then placed in the center of bigger beaker containing phosphate buffer pH 6.8 just touching the mucosal surface. The tablet was stuck to the lower side of balance pan and the platform was slowly raised until the tablet surface came in contact with mucosa. After a preload time of 5 minutes, water was added to the polypropylene bottle until the tablet was detached from the buccal mucosa. The water collected in the bottle was measured and expressed as weight (g) required for the detachment.

Dissolution studies:
The dissolution of the buccoadhesive tablets was performed in 500 ml of phosphate buffer (pH 6.8) using the USP dissolution apparatus II (Copoly Scientific, England) at 37 ± 0.5°C and 50 rpm. At appropriate time intervals, 5 ml of samples were withdrawn and an equal volume of medium was added to maintain the volume constant. Samples were filtered through a 0.45 µm millipore filter and suitably diluted, the amount of dilatiazem HCl which was released determined spectrophotometrically at 237nm and the release data were evaluated kinetically.

FTIR Study:
The buccoadhesive tablet (A32) were compressed and powdered. The palletized powder, along with KBr, was used for FTIR studies. The IR spectra were recorded using an IR-spectrophotometer (Shimadzu, Japan).

Results and discussion:
Carbopol (CP), sodium alginate (SA) and hydroxypropylmethyl cellulose (HPMC) polymers were selected owing to their excellent bioadhesive strength [3, 13], release rate controlling ability, non-toxicity, non-irritancy, stability at different pH ranges and compatibility with the drug. Successful use of the polymer combination of anionic polymer (like CP, SA) and a nonionic polymer
(like HPMC) is known to provide the formulation with controlled drug release along with desired mucoadhesive properties \[^{14}\].

**Physical properties of mucoadhesive Tablets:**

All the formulations showed acceptable hardness, friability and uniformity of content \[^{9}\] as shown in the table 2. Hardness of tablets was optimized on the basis of trial preparation of tablets. Hardness of tablets was maintained in the range of 3.5-5 kg/cm\(^2\) with SA/HPMC and 4-6 kg/cm\(^2\) with CP/HPMC formulas. Percentage weight loss in the friability test was found to be less than 1 % in all the formulations. The drug contents were also within limit for all formulations ranging from 96.97 % - 101 % \[^{10}\]. The surface pH of all formulation was found to be near the neutral pH as shown in table 2 and hence these formulations did not cause any irritation to the mucus membrane when applied \[^{15}\].

**Swelling studies:**

Adequate swelling behaviour of a buccal adhesive system is an essential property for uniform and prolonged release of drug and effective mucoadhesion \[^{16}\]. the swelling as well as the release of diltiazem HCl from buccoadhesive tablets varied according to the type and ratio of the matrix forming polymers. Swelling index of buccoadhesive tablets as a function of time was shown in Figure 1 and 2. The rate and extent of swelling increased with an increasing concentration of polymers in the formulations due to more gel forming abilities of polymers. The formulas A1 and A2 showed decrease in swelling index after a time which indicates the erosion of the polymer \[^{15}\]. Also, it has been shown that higher swelling was observed in formulas containing SA/HPMC. This result agreed with that obtained by Choi and Kimal \[^{13}\].

**Bioadhesive properties:**

The term "bioadhesion" is defined as an adhesion to biological surface and when adhesion occurs between the polymer and mucus layer only then it is referred as mucoadhesion. In general, mucoadhesion is considered to occur in three stages: wetting, interpenetration and mechanical interlocking \[^{2}\]. The degree of swelling of bioadhesive polymers is an important factor affecting adhesion. Adhesion occurs shortly after the beginning of swelling. Uptake of water results in relaxation of the originally stretched entangled or twisted polymer chains, resulting in exposure of all polymer bioadhesive sites for bonding to occur. The faster swelling of the polymer, the faster initiation of diffusion and formation of adhesive bonds \[^{18}\].

All formulations showed good mucoadhesive performance with mucoadhesion resistance time range from 5 hours for A1 to more than 12 hours for A31, A32, A33 and all B formulations. The bioadhesive strength for the prepared buccoadhesive tablets were showed in figure 3. It was revealed that increasing the polymer amount increased bioadhesive strength due to providing more adhesive sites and polymer chains for interpenetration with mucin \[^{19}\]. Also, the buccal tablets formulated with CP/HPMC (B1 to B4) showed stronger
mucoadhesion than SA/HPMC formulations (A1 to A4). This may be due to ability of CP to form secondary bioadhesion bonds with mucin and interpenetration of the polymer chains in the interfacial region, while other polymers, SA and HPMC undergo only superficial bioadhesion [17, 20].

It also showed that increasing in the HPMC/SA ratio from 1:1 (A31) to 2:1 (A32) and 3:1 (A33) increased the mucoadhesive strength which due to the hydrosolubility of HPMC, despite its moderate swelling properties, promoted liquid entry and entrapment in the polymer network [20].

**Dissolution studies:**

The in vitro drug release data obtained over a period of 8 hours, as expected, the drug release was significantly (p < 0.05) decreased with increasing in polymer content when 18.75 %, 37.5 %, 56.25 % and 75 % of SA/HPMC incorporated into formulations. The released amount of diltiazem HCl decreased from 97.5 % to 75.3 %, 66.65 % and 35.24 %, respectively at the end of 5 hours as shown in the figure 4. CP/HPMC formulations showed similar results for the same concentrations. The amount released was decreased in 6 hours from 46.87 % to 42.23 %, 30.9 % and 28 %, respectively, as shown in the figure 5. These results of study were consistent with the finding in previous report by Yamsant et al [21] which showed that an increase in the polymer concentration not only causes increase in the viscosity of the gel but also leads to formation of gel layer with a longer diffusional path. This leads to a decrease in the diffusion of the drug and therefore a reduction in the drug release rate.

The formulations containing CP/HPMC (B1, B2, B3 and B4) showed incomplete drug release (which was less than 60 %) within 8 hours compared with SA/HPMC formulations. It was reported for Carbopol that there are acid weakening inductive effects of ionized carboxylate residues that affect the ionization potential of neighbouring groups. This may lead to high coiling and proximity of carboxylic groups compare with linear polymer (SA) which leads to intramolecular hydrogen bonding. The crosslinking of Carbopol affects also elasticity of the chains as water penetrates inside the polymer network and this leads to entrapment of the drug inside the cross linked network of the polymer [22,23].

Also, it was revealed that increase the ratio of HPMC in HPMC/CA formulations from 1:1 (A31) to 2:1 (A32) and 3:1 (A33) was significant (p < 0.05) decrease the release rate from 91.4% to 80.11 % and 73.3 %, respectively, at the end of 8 hours as shown in figure 4 .This may be due to the increased viscosity produced by the gelling of the hydrophilic HPMC polymer [4, 24].

In order to describe the kinetics of drug release from controlled release preparations, various mathematical equations have been proposed (i.e zero, first, Higuchi and Hexon- Crowel equations). Furthermore, in order to better characterize the drug release mechanisms for the polymeric systems studied, the Korsmeyer-Peppas semi-empirical model was applied:

\[ \frac{Q_t}{Q_{\infty}} = K \cdot t^n \]
Where \( Qt/Q\infty \) is the fraction of drug released at time t, \( k \) constant compromising the structural and geometric characteristics of the device, and \( n \) the release exponent, which is indicative of the mechanism of drug release \cite{25}. For the case of cylindrical geometries such as tablets, \( n=0.45 \) corresponds to a Fickian diffusion release (Case I), \( 0.45<n<0.89 \) to a non-Fickian (Anomalous) transport, \( n = 0.89 \) to a zero order (Case II) release kinetics and \( n>0.89 \) to a super Case II transport \cite{25}.

The release exponent (Table 3) in all formulation is significantly greater than 0.5, which indicates anomalous (non-Fickian) drug release. When liquid diffusion rate and polymer relaxation rate are of the same order of magnitude, anomalous or non-Fickian diffusion is considered \cite{12, 26}. The value of \( n \) was greater in tablets containing SA-HPMC than that containing CP-HPMC. This observation could be attributed to the high swelling nature of alginate polymer which is in accordance with the higher swelling indices observed for these formulations.

The linear nature of the curves obtained for zero-order, first order, Higuchi model and Hixon-Crowel model as demonstrated by very close and higher r squared values Table 3 suggests that the release from the formulations may follow any one of these models. When the higher correlation coefficient values are considered, the release data seem to fit better with the zero order kinetics Table 3. Therefore, the release rate \( \mathrm{d}Q/\mathrm{d}t = k_0 \) is independent on its concentration or amount of drug incorporated in the formulation which could be considered as an advantage for fabricated systems.

The same mechanism of drug release was seen when verapamil hydrochloride, a water soluble drug, was formulated in hydrophilic matrix tablet \cite{12} and also when cinnarazine, a water soluble drug, was formulated in hydrophilic matrix tablet \cite{27}.

Figure 6 showed the FT-IR studies, the characteristic bands for important functional groups of pure drug, and tablet were observed without any change in their position indicating no chemical interaction between the drug and other polymer.

**Conclusion:**

From the results of present investigation, it may be concluded that sodium alginate / HPMC polymers are suitable for developing buccoadhesive tablet of diltiazem HCl. Formulation containing higher HPMC over SA exhibit higher mucoadhesion strength, swelling index and sustained release pattern. Thus, the study revealed that buccoadhesive formulation (A32) showed good mucoadhesion properties with sustain released of diltiazem HCl for more than 8 hours.
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Acknowledgements:
The author is very thankful to Mrs. Ghaida Al-Bayeti (M.Sc. pharmaceutics), College of Pharmacy, University of Al- Mustansiriya for her great help in supplying diltiazem HCl.

<table>
<thead>
<tr>
<th>ingredients(mg/tab)</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diltiazem HCl</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Sodium alginate</td>
<td>15</td>
<td>30</td>
<td>45</td>
<td>30</td>
<td>22.5</td>
<td>60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbopol</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>HPMC</td>
<td>15</td>
<td>30</td>
<td>45</td>
<td>60</td>
<td>67.5</td>
<td>60</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>PVP</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mannitol q.s to</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
</tr>
</tbody>
</table>

Table-1: Formulation of Diltiazem hydrochloride Buccoadhesive Tablets Prepared.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Thickness (mm)</th>
<th>Hardness (kg/cm²)</th>
<th>friability</th>
<th>% drug content</th>
<th>Surface pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>3.74 ± 0.023</td>
<td>3.6 ± 0.321</td>
<td>0.29</td>
<td>101 ±</td>
<td>6.66 ± 0.055</td>
</tr>
<tr>
<td>A2</td>
<td>3.34 ± 0.009</td>
<td>4.7 ± 0.642</td>
<td>0.15</td>
<td>0.56</td>
<td>6.61 ± 0.017</td>
</tr>
<tr>
<td>A3</td>
<td>3.31 ± 0.063</td>
<td>5 ± 0.5</td>
<td>0.154</td>
<td>98.87 ±</td>
<td>6.49 ± 0.015</td>
</tr>
<tr>
<td>A32</td>
<td>3.63 ± 0.023</td>
<td>4.6 ± 0.212</td>
<td>0.14</td>
<td>1.15</td>
<td>6.75 ± 0.051</td>
</tr>
<tr>
<td>A33</td>
<td>3.47 ± 0.005</td>
<td>4.8 ± 0.353</td>
<td>0.12</td>
<td>100.17 ±</td>
<td>6.93 ± 0.05</td>
</tr>
<tr>
<td>A4</td>
<td>3.49 ± 0.011</td>
<td>4.6 ± 0.577</td>
<td>0.29</td>
<td>1.36</td>
<td>6.66 ± 0.057</td>
</tr>
<tr>
<td>B1</td>
<td>3.62 ± 0.021</td>
<td>4.2 ± 0.404</td>
<td>0.36</td>
<td>99.26 ±</td>
<td>5.99 ± 0.018</td>
</tr>
<tr>
<td>B2</td>
<td>3.77 ± 0.005</td>
<td>5.4 ± 0.361</td>
<td>0.096</td>
<td>0.64</td>
<td>6.03 ± 0.03</td>
</tr>
<tr>
<td>B3</td>
<td>3.96 ± 0.0492</td>
<td>5.13 ±</td>
<td>0.12</td>
<td>96.97 ±</td>
<td>6.1 ± 0.017</td>
</tr>
<tr>
<td>B4</td>
<td>3.81 ± 0.005</td>
<td>0.321</td>
<td>0.062</td>
<td>0.72</td>
<td>5.8 ± 0.16</td>
</tr>
</tbody>
</table>

Table-2: Physical Properties, Surface pH of Diltiazem HCl Buccoadhesive Tablets.
Table-3: Correlation coefficient ($r^2$) of different models, drug release exponents (n), zero-order release rate constants ($k_0$), and MDT of different formulations of buccoadhesive diltiazem HCl tablets in phosphate buffer pH 6.8.

![Figure-1: Swelling profile of sodium alginate / HPMC formulations.](image)

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Figure-2: Swelling profile of carbopol / HPMC formulations.

Figure-3: In vitro bioadhesion strength of diltiazem HCl buccoadhesive tablets.
Figure-4: Release profile of diltiazem HCl from buccoadhesive tablet containing SA/HPMC at phosphate buffer pH 6.8.

Figure-5: Release profile of diltiazem HCl from buccoadhesive tablet containing CP/HPMC at phosphate buffer pH 6.8.

64
Figure-6: FTIR spectra of (A) diltiazem HCl (B) diltiazem HCl buccal tablet (A32).

References:


الخلاصة:

تم تحضير التنيدازول كليوباترات شرجية لغرض جعل الدواء متوفرا داخل الجسم عن طريق آخرى غير الفم، وبذلك يمكن تجاوز الآثار الجانبية للدواء على النهاية الوضيعة، وكذلك من الممكن استعمال هذا الشكل الدوائي عند صعوبة اعطاء الدواء عن طريق الوريد.

لقد تم دراسة تأثير نوع القاعدة اللوبوسية وتأثير المواد المخفضة للشد السطحي المضافة على الخواص الفيزيائية وعلى قابلية تحرر التنيدازول من اللوبوستات الشرجية باستخدام الواعدة الشحمية (الوايتيسول 15 والوايتيسول 35) والقواعد النانثة في الماء [خليل من البولي اثيلين كلاكيول 6000 : 400 (30 : 35) و 4000 : (30 : 70)].

لقد أوصت النتائج أن قابلية تحرر التنيدازول أكثر من القواعد النانثة في الماء مقاومة بالقواعد الشحمية والسبب في ذلك يعود ربما إلى وجود احتاد بين الدواء والتراكيزيرادات الموجودة في تلك القواعد الشحمية. كذلك وجد ان استخدام المواد المخفضة للشد السطحي قد زاد من تحرر التنيدازول من اللوبوستات الحاوية على الوايتيسول 15، وكانت الزيادة أكبر في حالة استخدام السباع 80 مقاومة بالتروين 80. كذلك تم دراسة تأثير درجة الحرارة وقطرة الخزن على الصفات الفيزيائية وقابلية تحرر الدواء بواسطة خزن اللوبوستات الشرجية لمدة 1، 15، 30 و 45 يوم بدرجتي حرارة 25 و 4 °C.

لقد أوصت النتائج أن لدرجة حرارة الأدنى تأثير على قابلية تحرر التنيدازول من اللوبوستات الحاوية على الوايتيسول 15 دون أن تؤثر على الخواص الفيزيائية لهذه اللوبوستات.

أما فترة الخزن فقد كان لها تأثير على كل من الخواص الفيزيائية وقابلية تحرر التنيدازول من هذه اللوبوستات.

من ناحية أخرى، فقد وجد أن لدرجة حرارة الأدنى تأثير على كل من قابلية تحرر التنيدازول والخواص الفيزيائية اللوبوستات الحاوية على البولي اثيلين كلاكيول بينما لم تكن لفترة الخزن أي تأثير على هذه اللوبوستات.

واخيراً، وجد أن مدة صلاحية التنيدازول في اللوبوستات الحاوية على البولي اثيلين كلاكيول 4000 : (30 : 70) تبلغ حوالي 3.3 سنة.
Abstract:
Tinidazole was prepared as a suppository dosage form that could be used rectally in an attempt to render the drug available systemically by a route other than oral route, thus overcoming the side effects of the drug on the gastrointestinal tract. Also such dosage form could replace the intravenous administration whenever it is inconvenient.

The influence of the type of suppository base and added surfactants on the physical properties and drug release was studied using lipophilic bases (witepsole H-15, witepsole H-35) and water soluble bases [mixtures of polyethylene glycols PEG 6000:400:200 (30:35:35) and PEG 4000:1000 (30:70)].

The results showed a good release of tinidazole from PEG bases compared to lipophilic bases because of the interaction between the drug and triglycerides of the lipophilic bases. Also the non ionic surfactants increase the release of tinidazole from witepsole H-15 base containing suppository and the increase was greater for span 80 than tween 80.

Also the influence of temperature and storage period on the physical properties and release was investigated by storing the prepared suppositories at 4°C and 25°C for 1, 15, 30 and 45 days. The results appeared that the temperature of storage affect the release of tinidazole from witepsole H-15, but had no effect on physical properties of the suppositories. While the storage period at both temperatures had some effects on the physical properties. On the other hand, temperature of storage affects the physical properties of polyethylene glycol suppositories and release of the drug, in contrast to the period of storage, which had no effect.

The expiration date of tinidazole in formula containing polyethylene glycol 4000:1000 (30:70) was found to be about 3.3 years.

Keyword: Tinidazole, suppositories, rectal preparations, suppository base.

Introduction:
Rectal preparations are intended for rectal use in order to obtain a systemic or local effect or may be intended for diagnostic purposes [1]. The suppository is a medicated solid dosage form generally intended for use in the rectum, vagina and to a lesser extent, the urethra[2]. They disintegrate in the body cavity either by melting or by dissolution [3]. The rectal route has many advantages such as it can avoid the hepatic first pass effect, and avoid the undesirable effect of meals on drug absorption[4, 5]. In addition the rectal route is useful in decreasing gastrointestinal side effects as found for Aspirin[6]. It is also preferred in the treatment of patients who are unable to make use of oral route. This may be the case when the patient has a problem in the gastrointestinal
tract like nausea and vomiting episode or case of unconsciousness \cite{4,7}. Furthermore, several categories of the patients, i.e. the very young, the very old or the mentally disturbed, may more easily use the rectal than the oral route \cite{8}. The drug may be insufficiently stable at the pH of the stomach or susceptible to enzymatic attack in the gastrointestinal tract\cite{9}. The suppository dosage form used to avoid unpleasant tasting or smelling drugs whose oral use is limited as metronidazole \cite{10}.

Tinidazole, like other structurally related drug metronidazole, demonstrate activity against the following protozoa: *Trichomonas vaginalis*, *Giardia lamblia* and *Entamoeba histolytica*. It also has a prophylactic use to prevent post operative anaerobic infections and used for the eradication of Helicobacter in peptic ulcer diseases \cite{11}.

The aim of this study is to formulate tinidazole as suppository dosage form, which suggested to be used rectally to obtain systemic effect in order to replace the intravenous route and to avoid the side effects of tinidazole on the gastrointestinal tract when used orally.

**Materials and Methods:**

**Materials:**

Tinidazole (Sigma chemical Co.), witepsole H-35 (supplied by Sammara Drug Industry SDI), witepsole H-15 (supplied by Al Shahbaa Pharma, Syria), PEG 4000 and PEG 200 (Hopkin &Williams, England), PEG 6000, PEG 1000, PEG 400, Potassium dihydrogen ortho-phosphate (BDH chemicals, Ltd. Pool, England), tween 80 (Merck schuchardt, Germany), Disodium hydrogen phosphate (E.Merck Darmstadt, West Germany), Span 80 (Atlas-chemie, Germany).

**Instruments:**

Balance (Sartorius AG, Gottingen, Germany), Dissolution apparatus (Copley, type FH 16-D, Nottingham, England), pH meter (pH 211, microprocessor, Italy), Hardness tester, Softening time tester (Erweka, Apparatteban GMBH, SBT, West Germany), UV visible spectrophotometer (Carrywin UV, Varian, Australia), suppository moulds 2 gm (stainless steel, ERBO Prazision Formenbau, GMPHD-7470 Albstadt 3).

**Methods:**

Preparation of tinidazole suppositories:

Tinidazole suppositories were prepared by fusion method in which 500 mg tinidazole and the surfactant (when used) were incorporated into the suppository base after it was melted by gentle heating on a water bath. The amount of the base used after calculating the displacement value. The melted mass was stirred constantly but slowly to avoid air entrapment, then the mixture poured into a 2 gm suppository
mold and then cooled in a refrigerator maintained at 5 °C. After that, any excess suppository mass was removed from the mold by scraping and then the mold was opened and the suppositories were removed. For suppository containing mixture of polyethylene glycols, the higher molecular weight polyethylene glycol was first melted, then the lower molecular weight polyethylene glycol were added and mixed well.

**Formulations:**

Different formulas were prepared using different types of bases and surfactants as shown in (Table-1) the displacement value of tinidazole in these bases was first determined and the amount of the base needed was calculated.

**Physical properties of the prepared suppositories:**

**Melting time determination:**

The suppositories were placed in a glass tube (2.5 cm diameter); 2 ml of Sorensen's phosphate buffer of pH 7.4 was then added. The tube was placed in water bath at 37°C. The time required for each suppository to melt completely or to disintegrate was determined.

**Hardness test (Resistance to rupture):**

This test determines, under defined conditions, the resistance to rupture of suppositories measured by the mass needed to rupture them by crushing. This test carried out using the Erweka hardness tester. The temperature inside the testing chamber was controlled at 25°C by means of circulating water from thermostat connected to the tester. The suppository was placed into the holding device with the tip upwards and the test chamber was then closed with glass plate. At this point, the initial load, which was given by the entire suspended block, was 600 gm. After one minute a disk of 200 gm was added and this weight addition was continued every minute until the suppository crush under the load of the weight.

The mass required to crush the suppository was calculated by the sum of the masses weighing on the suppository when it was collapsed (including the initial mass of the device i.e. 600 gm).

**Softening time test (for lipophilic suppositories):**

The softening time test indicates how long certain preparation takes to lose its physical structure. The suppository was inserted in the spiral shaped glass basket of the test tube with the tip pointed upwards and the tube was then closed. A thermostat connected to the tester provided circulating distilled water inside the test tube at the constant temperature 37°C and constant flow rate. The time required for the first drop of the suppository base to appear floating on the surface of the water inside the testing tube was considered as softening time.
In vitro dissolution test:
The dissolution rate of tinidazole from suppository was determined using a rotating basket dissolution apparatus at 50 rpm and at a constant temperature of 37°C. The medium was 900 ml of Sorensen's phosphate buffer solution of pH 7.4. At appropriate time intervals (0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes), 5 ml samples were withdrawn through syringe Millipore filter and the amount of tinidazole was determined by ultraviolet spectrophotometer at 310 nm.

Factors affecting the formulation:
Effect of type of suppository base:
Lipophilic bases [witepsole H-35 (formula 3) and witepsole H-15 (formula 4)] and hydrophilic bases [PEG 6000:400:200 (30:35:35) (formula 1) and PEG 4000:1000 (30:70) (formula 2)] were used to investigate the influence of the type of suppository base on physical properties and dissolution rate of tinidazole from the prepared suppositories.

Effect of type of surfactant:
The effect of type of surfactant on the physical properties and the dissolution rate of tinidazole from the prepared suppositories were studied by incorporating 5% span 80 and 5% tween 80 with witepsole H-15 containing suppository [formula (5) and formula (6), respectively].

Effect of storage time and temperature on tinidazole release and physical properties of the selected suppositories:
Experiments were conducted for studying the effect of storage time and temperature on the release of tinidazole and physical properties of different suppository formulas. The study was carried out using suppositories stored for 1, 15, 30 and 45 days at 4°C and 25°C. Two formulas were selected for this study [formula (2) and (5)]. The suppositories were wrapped with aluminum foil, placed in tightly closed containers and stored at the mentioned temperatures for the periods indicated [2].

Finally, formula (2) was stored for 30, 60, 120 and 180 days at 25°C to determine the shelf life.

Results and Discussion:
Factors affecting the formulation of tinidazole suppositories:
Effect of suppository base:
The effect of the type of the base on the physical properties of the prepared suppositories was illustrated in (Table -2). It appears that all the suppositories were within the limits recommended by the British Pharmacopoeia (disintegration occurs within 30 minutes for fat based suppository and less than 60 minutes for water soluble suppository). Also it was found that hydrophilic bases (polyethylene glycol) have long
melting time than oleaginous bases (witepsole). Beside that the hardness of the suppositories prepared from polyethylene glycol was found to be less than that observed for oleaginous bases [14].

Figure (1) illustrates the effect of changing the suppository base type on the in vitro release of tinidazole from the prepared suppositories. It appears that the amount released was higher from hydrophilic bases [formula (1) and (2)] when compared with the oleaginous bases [formula (3) and (4)]. This is due to the lower water solubility of tinidazole, so the affinity of the drug to lipophilic bases is higher than the hydrophilic bases which make the entrapment of the drug within these bases easy. On the other hand, the high release percentage of tinidazole from hydrophilic bases may be related both to the affinity of the drug for these bases, and the water solubility of the base in the aqueous medium (i.e. the solubility of the drug in the base greatly influence the amount of the drug released from those suppositories). The results are in consistence with the results obtained in the formulation of flurbiprofen [16] as suppository dosage form.

Also there was a slight increase in the release of tinidazole from formula (2) as compared with formula (1). This may be due to the fact that the release from polyethylene glycol base was found to be increased as molecular weight decreased [17].

Changing the type or ratio of polyethylene glycol mixture from formula (1) to (2) affect the physical properties of the suppositories due to the fact that the melting point and hardness of polyethylene glycols increase as a function of polymerization of the polymer used, that increase with the molecular weight used [1].

**Effect of addition of surfactants to the suppository base:**

Non ionic surfactants were used in an attempt to improve the release of tinidazole from the suppository [18]. This was achieved by incorporating 5% (w/w) span 80 or tween 80 in witepsole H-15 [formula (5) and (6) respectively.

Witepsole H-15 was chosen because it was found to be the base of choice for use in countries of continental climate [19].

The addition of these non ionic surfactants resulted in an increase in the amount released of tinidazole from witepsole H-15 suppository base as shown in figure (2). This could be due to an increase in the wetting and spreading properties of the base and subsequent increase in the dissolution rate of the drug [20] also may be due to the micellar solubilization of the drug by surfactants [19]. Moreover, surfactants decrease the disintegration time and thus increase the release of active ingredients from suppository base [21].

It was found that span 80 (formula 5) produced the highest release compared with tween 80 (formula 6). This could be due to the HLB
(hydrophilic-lipophilic balance) value of span 80 which is equal to 4 which is considered to be the optimal HLB value for surfactant incorporated in suppository base [22].

Non ionic surfactants also affect the physical properties of the prepared suppository. It was found that the addition of these surfactants resulted in a decrease in the melting time, softening time and the hardness as shown in (Table -3). This could be due to the fact that these adjuvants are miscible with lipophilic excipients, so homogenous mixture of heterogeneous composition was obtained. Consequently, the melting temperature of the base-excepients was decreased [23].

Effect of storage time and temperature on tinidazole release and physical properties of the selected suppository:

Formula (2) and (5) were used in this study because of their higher release behaviors. Samples were selected from these formulas and stored for 1, 15, 30 and 45 days at 4°C and 25°C. (Table -4) showed that there was a reduction in the melting time and hardness of formula (2) tinidazole suppository [PEG 4000:1000 (30:70)] stored at 4°C, especially after 30 and 45 days of storage, with slight changes in these parameters on storage at 25°C. This observation can be explained by the fact that storing polyethylene glycols based suppositories at low temperatures cause an increase in their brittleness with subsequent decrease in melting time and hardness [24].

The effect of the storage conditions at different temperatures on the release behavior of tinidazole from the selected formulas was studied. For formula (2) which was stored at 4°C, showed an increase in the release rate for samples stored at 45 days compared to those tested after one day of preparation, as presented at figure (3). This may be due to an increase in their brittleness which makes the drug rapidly released from the suppository [24].

While figure (4) represent the effect of storage time and temperature on the release of tinidazole from formula (2) suppositories stored at 25 °C. It shows no significant changes in the release rate for samples stored at different time intervals since the base is stable at 25 °C and undergo no brittleness [24].

On the other hand, formula (5) shows an increase in the melting time and hardness on storing at different time intervals at 25°C with no significant changes at 4 °C as shown in (Table -5). This may be due to the crystallization of witepsole base at room temperature when stored for prolong time [25].

For the same reason, there was a decrease in the release rate of tinidazole from formula (5) stored at 25 °C for samples tested after 45 days compared to ones tested soon after preparation due to the crystallization of the witepsole bases when stored at room temperature for
prolong time \textsuperscript{[25]} as shown in figure (6). While figure (5) illustrates that there was no significant change in the release rate of tinidazole from formula (5) at 4 °C.

**Shelf life study:**
To evaluate most promised formula for tinidazole rectal suppository, formula (2) was introduced to study the shelf life. The suppositories were stored at 25°C and samples analyzed for drug content at 30, 60, 120 and 180 days.

The degradation of tinidazole followed first order kinetics since the plot of the logarithm of percent remaining of tinidazole versus time gave straight line \textsuperscript{[26]} as shown in figure (7). The degradation rate constant of tinidazole at 25°C (K_{25°C}) was determined and found to be (0.4 \times 10^{-4}) day\(^{-1}\). The shelf life then was calculated from the following equation and found to be 3.3 years.

\[ t_{10\%} = \frac{0.105}{K_{25°C}} \]

**Conclusion:**
The release of tinidazole from water soluble bases was better than lipophilic bases. The non ionic surfactants improve the release from witepsole H-15 base especially for span 80 and to a lesser extent tween 80.

The storage time and temperature affect both the physical properties of the suppositories and the release of tinidazole. The expiration date of tinidazole in suppository dosage form was about 3.3 years.

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>Base</th>
<th>Tinidazole 500 mg</th>
<th>Span 80 (w/w)</th>
<th>Tween 80 (w/w)</th>
<th>Displacement value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PEG 6000:400:200 (30:35:35)</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>1.19</td>
</tr>
<tr>
<td>2</td>
<td>PEG 4000:1000 (30:70)</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>Witepsole H-35</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>1.45</td>
</tr>
<tr>
<td>4</td>
<td>Witepsole H-15</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>Witepsole H-15</td>
<td>500</td>
<td>5%</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>Witepsole H-15</td>
<td>500</td>
<td>-</td>
<td>5%</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table-1: Composition of tinidazole suppositories using different bases and surfactants and the calculated displacement values
<table>
<thead>
<tr>
<th>Formula no.</th>
<th>Base type</th>
<th>Melting time (min.)</th>
<th>Softening time (min.)</th>
<th>Hardness (Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PEG 6000:400:200 (30:35:35)</td>
<td>33</td>
<td>–</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>PEG 4000:1000 (30:70)</td>
<td>31</td>
<td>–</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>Witepsole H-35</td>
<td>13</td>
<td>6</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>Witepsole H-15</td>
<td>11</td>
<td>5</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table-2: Effect of changing the type of base on the physical properties of tinidazole suppositories.

<table>
<thead>
<tr>
<th>Formula no.</th>
<th>Base type</th>
<th>Melting time (min.)</th>
<th>Softening time (min.)</th>
<th>Hardness (Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Witepsole H-15</td>
<td>11</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>Witepsole H-15 + 5% span 80</td>
<td>9</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>6</td>
<td>Witepsole H-15 + 5% tween 80</td>
<td>10</td>
<td>5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Table-3: Effect of addition of surfactant on the physical properties of tinidazole suppositories.
Storage time (days) | parameters | Parameters | 4°C | 25°C |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melting time (min.)</td>
<td>Hardness (Kg)</td>
<td>Melting time (min.)</td>
<td>Hardness (Kg)</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
<td>2.8</td>
<td>31</td>
<td>2.8</td>
</tr>
<tr>
<td>15</td>
<td>30</td>
<td>2.6</td>
<td>32</td>
<td>2.6</td>
</tr>
<tr>
<td>30</td>
<td>28</td>
<td>2.2</td>
<td>29</td>
<td>2.7</td>
</tr>
<tr>
<td>45</td>
<td>24</td>
<td>2.0</td>
<td>30</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table-4: Effect of storage time and temperature on the physical properties of tinidazole suppository formula (2) at 4°C and 25°C.

Storage time (days) | parameters | Parameters | 4°C | 25°C |
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Melting time (min.)</td>
<td>Hardness (Kg)</td>
<td>Melting time (min.)</td>
<td>Hardness (Kg)</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>2.6</td>
<td>9</td>
<td>2.6</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>2.5</td>
<td>9</td>
<td>2.6</td>
</tr>
<tr>
<td>30</td>
<td>9</td>
<td>2.5</td>
<td>10</td>
<td>2.7</td>
</tr>
<tr>
<td>45</td>
<td>10</td>
<td>2.6</td>
<td>12</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Table-5: Effect of storage time and temperature on the physical properties of tinidazole suppository formula (5) at 4°C and 25°C.

Figure-2: Effect of type of surfactant on the in vitro release of tinidazole from the prepared suppositories in Sorensen's phosphate buffer of pH 7.4 at 37 °C. (formula 4: Witepsole H-15, formula 5: Witepsole H-15 + span 80 (5% w/w), formula 6: Witepsole H-15 + tween 80 (5% w/w)).
Figure-3: Effect of storage time and temperature on the release of tinidazole from formula (2) suppositories stored at 4°C using Sorensen's phosphate buffer of pH 7.4 at 37 °C.

Figure-4: Effect of storage time and temperature on the release of tinidazole from formula (2) suppositories stored at 25°C using Sorensen's phosphate buffer of pH 7.4 at 37 °C.
Figure-5: Effect of storage time and temperature on the release of tinidazole from formula (5) suppositories stored at 4°C using Sorensen's phosphate buffer of pH 7.4 at 37 °C.

Figure-6: Effect of storage time and temperature on the release of tinidazole from formula (5) suppositories stored at 25°C using Sorensen's phosphate buffer of pH 7.4 at 37 °C.
Figure-7: Determination of the expiration date of tinidazole in formula (2) suppositories stored at 25°C.

References:


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The use of the water extract of *Rosa* spp petals as a bacterial growth medium

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Abstract:

In a pioneer study, simple water extract for the red petals of *Rosa* spp. was prepared under sterile conditions, then used for the first time as experimental bacterial culture medium for the growth of the bacteria: *Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Escherichia coli,* *Streptococcus pneumoniae* *and Klebsiella pneumoniae*. The medium was used as alternative culture medium for the routine culture media (Nutrient agar, MacConkey agar and blood agar) that used for the growth of these genera in the laboratories.

All the genera showed active growth after 24 hours when it used directly as a liquid culture medium. The extract was used also to enrich the agar-agar and cultivated with the same bacteria; it showed a noticeable growth. The results suggest that this extract is a suitable culture medium; it could be used instead of the routine culture media that used in the cultivation of these bacteria in the laboratories.
laboratories. It also represents important, rich nutritional medium as those that is used in the routine laboratory work.

Introduction:

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The food base that supports the growth of an organism is called culture medium; the biochemical (nutritional) environment is made available in this culture medium\(^{[1-4]}\).

A growth medium is a mixture of nutrients, moisture and other chemicals that bacteria need for growth in a laboratory environment. Media can be solid, such as Jell-O-like agar that is poured into the bottom half of a Petri dish, or media can be liquid to allow for bacterial growth in test tubes \(^{[1-5]}\).

The food base depending upon the special needs of particular bacteria (as well as particular investigators), so that a large variety and types of culture media have been developed with different purposes and uses \(^{[1,2]}\). These include sources of organic carbon, nitrogen, phosphorus, sulfur and metal ions including iron \(^{[6]}\). Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties \(^{[1,3,4]}\).

The manner in which bacteria are cultivated, and the purpose of culture media, varies widely. Liquid media are used for growth of pure batch cultures, it include media may be made from animal tissue and fluids, e.g., nutrient broth, serum broth, carbohydrate broths, milk, blood, nitrate peptone solution, Dunham's solution; or from vegetable tissue such as malt extract (germinated barley), beer wort, yeast extract, hay infusion, natural fruit juices, wines (fermented fruit juices) \(^{[3,4]}\). Solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes; the usual gelling agent for solid or semisolid medium is agar, a hydrocolloid derived from red algae. Agar is used because of its unique physical properties (it melts at 100\(^\circ\)C and remains liquid until cooled to 40\(^\circ\)C, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aqueous solution \(^{[1,3,4]}\).

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis. The chemicals and elements of this environment that are utilized for bacterial growth are referred to as nutrients or nutritional requirements. Many bacteria can be grown in the laboratory in culture media which are designed to provide all the essential nutrients in solution for bacterial growth \(^{[1,3-5]}\).

Basically, the culture media are of three types: natural, synthetic and a complex (undefined) media. Natural medium is that which contains the natural products such as, for example diluted blood, urine, milk, vegetable juices,
peptone or animal cells/tissues/organs. In such medium the exact chemical composition is not known. A synthetic medium is one chemically-defined in which the exact chemical composition and concentration is known. A complex (undefined) medium is one in which the exact chemical constitution of the medium is not known \cite{1,5,7}.

Other concepts employed in the construction of culture media are the principles of selection and enrichment. A selective medium is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species. A culture medium may also be a differential medium if it allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium; MacConkeys & Mannitol Salt Agars in addition to being selective, they are also differential\cite{1,3-5,8-11}. An enrichment medium employs a slightly different twist. It contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment. However, an enrichment medium may have selective features \cite{1,3,5,8,10}. Blood agar is an enriched medium that provides an extra rich nutrient environment for microbes. It contains 5% sheep blood; therefore (BAP) is not a selective growth medium, since it supports the growth of a wide range of organisms\cite{3-5,8,11,12}. Nutrient agar is used for the cultivation of bacteria and for the enumeration of organisms in water, sewage, feces and other materials. It is used in the laboratory for the cultivation and maintenance of nonfastidious species \cite{3,5,7,11,12}.

In a pioneer study, a simple water extract for the red petals of *Rosa* spp. was prepared under sterile conditions, then used as an experimental culture medium to examine its ability to produce a suitable source of nutritional requirements for many types of (Gram+) and (Gram-) bacteria as a substitute culture medium for the routine bacterial culture media (MacConkey agar, blood agar and nutrient agar) that used for the growth of these types in the laboratories; the types of used bacteria were: *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae* and *Klebsiella pneumoniae*. In the first experiment the extract used directly as a liquid culture medium; in the second experiment the extract used to enrich the (agar-agar) and it used as a solid culture medium. It could be considered this medium (the extract) from the natural type of media because it is from natural source (vegetable juice), its exact chemical composition not known although the presence of some components is expected; such components are water, protein, lipids, aromatic oil, polysaccharides and salts \cite{13}. The availability of the suitable nutrients and the fitting of medium for the growth were investigated by the bacterial growth in the liquid and enriched solidified medium; the growth of these bacteria compared with their growth in the original media. The aim of this study is to find an alternative medium characterized by cheap cost and simple
preparation could be used instead of the routinely prepared media that is used in the laboratories to grow these types of bacteria.

Materials and Methods:

The preparation of the experimental medium (the extract):

The preparation of the extract done according to Al-Succary et al.\textsuperscript{13} with minor modification from Al-Azzauy\textsuperscript{14}; where the red petals of Rosa spp. were collected and washed carefully with tap water to remove the dust and other materials. Then (250)g of these red petals were putted in (1)L beaker and (250)ml of distilled water were poured in the beaker, the mixture were heated for (10)minutes in (100)°C. The extract refines by the refinery then cooled to room temperature, and then it is filtered by Whatman filter paper, the pH adjusted at (7.4). After then the extract were sterilized by millipore filter (0.22)µ, putted in sterile bottles and preserved in (4)°C to be ready for use then.

The use of the extract in the bacterial growth:

1- The extract as liquid growth medium

The extract were dispensed into many sterile universal tubes (2 ml in each one) and used as a liquid growth medium. The bacteria were selected from (24) hrs. incubated bacterial suspensions, turbidity was visually adjusted to that of (0.5) McFarland turbidity standard (1.5×10\textsuperscript{8} Colony Forming Unit (CFU)/ml. The extract inoculated with (0.1)ml of the bacterial suspensions of \textit{P. aeruginosa}, \textit{P. vulgaris}, \textit{S. aureus}, \textit{E. coli}, \textit{S. pneumoniae}, and \textit{K. pneumoniae} respectively, these types of bacteria were collected from patients and all the isolates were stained with Gram stain and diagnosed by the biochemical tests: urea, Simmon citrate, TSI, Indol and motility for (Gram-) bacteria (\textit{P. aeruginosa}, \textit{P. vulgaris}, \textit{E. coli} and \textit{K. pneumoniae}), and the tests: catalase, coagulase for (Gram+) bacteria (\textit{S. aureus}, \textit{S. pneumoniae}) and mannitol salt agar for \textit{S. aureus}\textsuperscript{15}. After (24) hrs of incubation at (37)°C, the bacterial growth, color of the medium and the pH of the medium were investigated. The intensity of growth measured by the turbidity, color and pH change. The viability of the bacteria investigated every day after the preliminary period of incubation (the 24 hrs.) by making subcultures, the purpose of this step were to estimate the ability of this medium in the maintenance of the bacteria.

2- The solidified growth medium (the extract as enrichment substance to the agar-agar)

After the preparation of agar-agar (Biolife company) it sterilized by autoclave then left to cool for (40)°C and enriched with the prepared extract; this is done by the dilution of (15)gm of agar-agar in (200)ml of distilled water followed by adding (800)ml of the extract. After then the medium poured into sterile Petri dishes then cultivated by spreading of (0.1)ml of bacterial suspensions(0.5) McFarland turbidity standard (1.5×10\textsuperscript{8} CFU/ml by the loop from the same types of bacteria mentioned above (\textit{P. aeruginosa}, \textit{P. vulgaris}, \textit{S. aureus}, \textit{E. coli}, \textit{S. pneumoniae}, and \textit{pneumoniae}) respectively. After
incubation at (37)°C for (24) hrs. the bacterial growth, the color of the medium and the colonies were investigated. The viability of the bacteria investigated every day after the preliminary period of incubation (the 24 hrs.) by making subcultures as mentioned above.

3- Examination of the growth cells (in the liquid medium) and the colonies (in the solid medium) using Gram stain:

After the incubation period and the appearance of the growth in the both experimental media (liquid medium and enriched solidified medium), the biochemical tests mentioned above were repeated for all the types of the used bacteria; as well as many samples were collected from each type of these experimental media for microscopic examination and stained with Gram stain to ensure that this growth was from the certain selected isolate and not because of any contamination.

Results:

After the incubation period, each type of media (the liquid and enriched solidified media) showed noticeable bacterial growth. The color and pH were changed in the liquid medium; they are synchronized with the average bacterial growth which is detected by the turbidity of the medium. All the used types of bacteria still viable in this medium for (7) days.

The color of the medium converted from red to yellow in the medium inoculated with *S. aureus*, *S. pneumoniae*, and *K. pneumoniae*, while it is converted to dark yellow, dark brown and light brown in the medium inoculated with *E. coli*, *P. aeruginosa* and *P. vulgaris* respectively, this is very explicit in the figures (2) and (3). The pH of the liquid medium was increased from (7.4) to (6.3) after the growth of *S. aureus*, *E. coli*, *S. pneumoniae*, and *K. pneumoniae* in the medium; while increased to (5.9) in the medium inoculated with *P. aeruginosa*, *P. vulgaris*. (Table-1) summarized these results.

The solidified enriched medium showed considerable bacterial growth but the color of medium not changed with growth. The color of the bacterial colonies was mostly transparent in *S. aureus*, *E. coli* and *S. pneumoniae*, while the colonies of *P. aeruginosa* were brown to grayish, and were grayish in *P. vulgaris* and *K. pneumoniae*, (Table -2). All the used types of bacteria still viable in this medium for (4-5) days.

After the staining of the slides which prepared from the growing isolates in both the liquid and solid media with Gram stain, the microscopic examination showed (Gram-) rods present as single bacteria or in pairs or in short chains in the slides prepared from the cultures of *P. aeruginosa*; in the slides prepared from the cultures of *P. vulgaris* there were (Gram-) rods, while in the slides prepared from *S. aureus* cultures there were (Gram+) cocci single, pairs, tetrads and chains arranged in irregular clusters. The microscopic examination of *E. coli* cultures showed short (Gram-) rods; the slides of *S. pneumoniae* showed
(Gram+) single cocci or pairs and the slides of *K. pneumoniae* showed (Gram-) rods with large capsule.

<table>
<thead>
<tr>
<th>Type of Bacteria</th>
<th>The color of liquid medium (the extract)</th>
<th>The pH of the liquid medium (the extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before growth</td>
<td>After growth</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Red</td>
<td>Dark brown</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>Red</td>
<td>light brown</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Red</td>
<td>Yellow</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Red</td>
<td>Dark Yellow</td>
</tr>
<tr>
<td><em>Streptococcus pneumonia</em></td>
<td>Red</td>
<td>Yellow</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Red</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

**Table-1:** The color and pH changes in the liquid medium (extract) before and after inoculation with different types of bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Color of colonies in solidified medium (the extract)</th>
<th>Color of colonies in MacConkey agar</th>
<th>Color of colonies in Blood agar</th>
<th>Color of colonies in Nutrient agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Brown to Grayish</td>
<td>-</td>
<td>-</td>
<td>Green</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>Grayish</td>
<td>-</td>
<td>-</td>
<td>White</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>transparent</td>
<td>-</td>
<td>Grayish</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>transparent</td>
<td>Pink</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>transparent</td>
<td>-</td>
<td>Translucent</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Grayish</td>
<td>-</td>
<td>-</td>
<td>White</td>
</tr>
</tbody>
</table>

**Table-2:** The color of the colonies in the solid medium (the solidified extract), MacConkey agar, blood agar and nutrient agar.

* The sign (-) mean that this medium was not used for the growth of this type of bacteria.
Figure-1: The liquid culture medium (the extract)

Figure-2: The liquid medium after the cultivation with before ultivation.
(from left side): S. aureus, S.pneumoniae, K. pneumoniae, P. aeruginosa and P. vulgaris.

Figure-3: liquid medium after cultivation with E. coli.

Figure-4: Solid enriched medium without cultivation.

Figure-5: Cultivated solid enriched medium.
Right: P.vulgaris.
Left: K. pneumoniae.

Figure-6: Cultivated solid enriched medium.
Up: P. vulgaris.
Down: P. aeruginosa.
Discussion:

The results suggest that the liquid medium (the extract) is a good substitute for the nutrient broth in the cultivation and maintenance of *P. aeruginosa*, *P. vulgaris*, *S. aureus*, *E. coli*, *S. pneumoniae*, and *K. pneumoniae*. This medium represents a suitable medium for the growth of all the used types of bacteria although it is from plant origin, this fittings very clear from the intensity of the growth that obtained from the inoculation of the medium with the above types of bacteria which measured by the turbidity, the color and pH change as it seen in (figures 2 and 3); moreover, the medium maintained all the used types of bacteria for at least (7) days with full viability. This is may interpreting as that this medium provides these types of bacteria with all the essential requirements for their growth.

This experimental medium has a red color because of the use of the red petals of *Rosa spp*. in the extraction; the color resulting from the natural stain that present in the cell juice of the petals' cells (Anthocyanin) which become free in the extract solution after the destruction of the cells by the heat during the preparation of the extract figure(1). This color changed after the bacterial growth in the medium which resulting in acidic metabolic products as a result of the fermentation in some of the extract's components by the bacteria; these products contributed in the change of the pH, and because the Anthocyanin color changing with pH, then the stain's color converted since the pH of the medium changed. This change gave a good indicator for the presence of the growth, as well as to the turbidity, from one hand and used as an evidence for the pH change (which then approved by the litmus paper) from the other hand; this character reduced the need for adding any dye to the prepared medium (Table-).

The solidified growth medium produced a suitable environment for the bacterial growth and met the nutritional requirements for the growth of these types of bacteria. The success of this medium in its job approved by the bacterial growth that obtained after the cultivation of the bacteria as it appears in the
figures (5, 6, 7 and 8). The growth of (Gram+) bacteria was less intensity in our prepared medium; nevertheless, *E. coli* (Gram- bacterium) showed a weak or limited growth in this medium figure(7), this limited growth of *E. coli* in the solidified enriched medium may interpreting as a result of absence in one or more essential substances for its growth in this medium.

The progressive growth of *P. aeruginosa, P. vulgaris* and *K. pneumoniae* in the enriched medium figure (5 and 6) interpreting as that the components of this experimental medium supplies these bacteria with its growth requirements, so it could be consider this medium very suitable for their growth and maintenance also since the growth of these types continue for at least (4-5) days in this medium, in addition, it could considered a good substitute for the nutrient agar which used for the growth of these types.

The chemical components of our enriched solidified medium is free of the animal protein, blood, serum and any other enriched components from animal origin which found in the blood agar (the suitable medium for *S. aureus* and *S. pneumoniae*), so that, there is a slight possibility of expediency of the prepared medium to the growth of these bacteria. In spite of that, there was a moderate growth of *S. aureus* in the enriched solidified medium, may be because this type of bacteria has a high resistance against unsuitable conditions; while the genus *S. pneumoniae* showed a limited growth, because this type needs a special medium meet their nutritional and environmental requirements and provide it with a suitable growth components that similar to those in their infected foci which is absent in this experimental medium.

The color of the enriched solid medium before the cultivation with any bacteria is brown (figure 4); it is not changed after the growth of each one of the six types. The color of the colonies of the certain type was different each from the other in the enriched medium, the colonies takes a color ranges between grayish (in *P. vulgaris* and *K. pneumoniae*) or brown to grayish (in *P. aeruginosa*) to transparent (in *S. aureus, E. coli* and *S. pneumoniae*), (Table-2).

The biochemical tests confirmed that the diagnosed samples are the same types of the selected bacteria, this step improve that the obtained growth was not because of any contamination in the medium or during the cultivation of the bacteria.

As a conclusion, it could use the liquid form of this extract as a liquid culture medium directly instead of the nutrient broth and get acceptable results. If the extract used as an enriched substance for the agar-agar it produces a suitable source for nutrients and promote the growth of the certain type of bacteria. It could not consider this medium as a selective medium because it provides a rich nutritional environment for many types of microbes since it supports the growth of many types of (Gram+) and (Gram-) bacteria. It also could not consider as a differential medium because it isn’t providing colonies with different color according to the type of the cultivated bacteria. Generally, the medium represents a cheap, simple prepared natural medium used for the
cultivation of different type of bacteria and it could substitute this medium instead of the routine media in the cultivation of many types of bacteria such the types used in this research.

**References:**

9. Baron, S. (1996). Baron's Medical Microbiology, Univ. of Texas Medical Branch, 4th ed, USA.
14. Al-Azzauy, A. A. M. (2002). The extraction of (RA) & (BA) plant stains and the use of them in the viability test of *Echinococcus granulosus* protoscolices, patent, registered by the central organization for standardization and quality control, Iraq-Baghdad, no. of patent 3031.
Evaluate the antibacterial effect of Garlic (*Allium sativum*) and antimicrobial susceptibility on *Pseudomonas aeruginosa* isolated from otitis media

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Abstract:

Twenty swab samples were taken from patients suffering from otitis infection; these samples were cultured on different agar media (blood agar base, macConkey and cetramide). Several microscopical, morphological and biochemical tests were performed and results showed that 50% of isolated colony were belonged to *Psudomonas aeruginosa*. In addition it was found that males were more susceptible to infection with otitis than female especially at teenager aged.
Five isolates were subject to sensitivity test against several antibiotic groups. Result showed that all five isolates were resistance to pencilline, tetracycline, Cefotaxin (100%) and (60%) of them showed resistance to cefotaxime and (40%) to chloroemphenicol and (20%) to amoxicillin while all isolates were sensitive to ciprofloxacin. The minimum inhibitory concentration (MIC) test was performed for two multiple drugs resistance isolates (P1 and P2) result declared that both P1and P2 showed high-level of resistance to Cefotaxime and chloroemphenicol with MIC (128µg/ml), followed by (64µg/ml) to ampicillin while P2 was (128 µg/ml), as well as, the amoxicillin showed the lowest MIC (16 µg/ml) forP1 and (64 µg/ml) to P2.

No pH effect was observed on the activity of garlic when antibacterial effect of garlic extract against Pseudomonas aeruginosa was investigated at different PH. The MIC of garlic extract on Pseudomonas aeruginosa at different incubation periods was determined. Results showed that after 24hrs MIC of extract was greater than 8µg/ml for P1 isolate and 16µg/ml for P2 isolate. While, after 72rs it was found that the MIC at concentrations (32, 64 and 128 µg/ml) were more effective in inhibition growth of (P1, P2) while in (8 µg/ml) that indicated heavy growth of P. aeruginosa

Introduction:

Otitis media (Latin for "Middle otitis") is inflammation of the middle ear, or middle ear infection. Otitis media occurs in the area between the ear drum (the end of the outer ear) and the inner ear, including a duct known as the Eustachian tube.\[1\]

Middle ear infection is mainly a problem in children, although it also occurs in adults. \[2\] declared that otitis media is one of most common disease of children which is the leading cause hearing loss in children and the most frequent indication for antimicrobial or therapy in children.

Pseudomonas spp. were the most commonly identified etiologic agents causing dermatitis, conjunctivitis, or otitis. In humans, P. aeruginosa is the second most frequent gram-negative nosocomial pathogen in hospitals.\[3\] P. aeruginosa is frequently found in the normal ear and is the predominant bacterial pathogen in some cases of external otitis. The bacterium can cause a more serious ear infection in elderly patients, possibly leading to hearing problems, facial paralysis, or even death. Ear infection of P. aeruginosa can cause infections in the external ear canal-- so-called swimmer's ear"-- that usually disappear without treatment.\[4\]

There is extensive literature on the antibacterial effects of fresh garlic juice, aqueous and alcoholic extracts, lyophilized powders, steam distilled oil and other commercial preparations of garlic. It is a broad spectrum antibiotic, killing a wide variety of bacteria. Many pharmaceutical antibiotics kill only a narrow range of these germs while garlic has the broadest spectrum of any antimicrobial substance that otitis know due to antibacterial, antifungal,
antiparasitic, antiprotozoan and antiviral. This property belongs to the garlic
constituent allicin, which is released when cutting garlic clove. Moreover,
garlic extracts exhibited activity against both gram negative (E. coli, Salmonella
sp. and Citrobacter, enterobacter, Pseudomona and Klebsiella) and gram
positive (S. aureus, S. pneumonia Group A Streptococcus and Bacillus anthcris)
all of which are cause of morbidity worldwide. The present study tested an
aqueous extract of dried garlic in vitro for its antibacterial activity against
Pseudomonas aeruginosa isolates isolated from otitis patients with
determination their susceptibility to antimicrobial agents.

Materials and Methods:
Bacterial isolation:
Twenty swab samples were obtained from Al-Yarmook Teaching
Hospital in Baghdad from the periods (1/9/2009-1/10/2009) from patients
suffering from otitis disease. Specimens were obtained by sterile cotton swab
these were processed for direct examination and cultivation on blood agar
media, MacConkeys agar and cetramide agar media (Banagalore, India) and
were identified initially as Pesudomonas species according to their
morphological, physiological and biochemical properties as indicated by
[7,8] depending on Gram stain, colony shape, oxidase, catalase, growth on cetramide
medium, production of pyocynine, growth at 4 and 42 °C.

Antibiotic susceptibility
The use of antimicrobial sensitivity test is essential for the selection of an
appropriate drug for treatment of otitis infection. The disc diffusion method was
used in this study against 5 isolates of Pseudomonas aeruginosa depending on
the Kirby-Bauer diffusion method [9]. Up to 7 different groups of discs of the
available antimicrobial agents were used in this study.

Minimum inhibitory concentration:
Broth microdilution method was performed, in this experiment twofold
dilutions of antibiotics were done in broth media and broth was inoculated with
105 CFU/ml of the tested organisms. After incubation for 18-24 hrs, MIC was
described as concentration in which no visible growth was observed. [8]

Preparation of garlic extract:
The garlic bulbs were washed thoroughly under tap running water
aseptically cut into small pieces with a knife and then kept in the shade for 1-3
days at 32-35°C. The semi-dried pieces were then crushed using pestle and
mortar, and left to dry in the shade at room temperature for further two days.
The dried garlic materials were further ground to powdery form with a Kenwood
blender. Two hundred gram (200.0 g) of garlic powder was extracted with 500
ml of solvents distilled water, for 24 h by using Soxhlet apparatus. The extract
were concentrated using a rotary evaporator at 40°C. Clearly prepared garlic
powder was thoroughly mixed with distilled water and the concentration was
determined with varying amounts of crude preparation of garlic to give the final
concentration of 8, 16, 32, 64 and 128 mg/ml of brain heart infusion broth media and the final volume of 10 ml.\cite{10}

**Results and Discussion:**

Isolation of *Pseudomonas* Species:

Twenty swab samples were collected from patients suffering from middle ear infection (otitis) (male and female) from Al-Yarmook teaching Hospital belongs to variety ages from (5-25) years. Results in table (3) showed that males were more susceptible to infection with otitis than female especially at teenager aged the reason for that is male have hospitalization rate ratio (HRR) for admission due to a respiratory tract infection more than female. Hospitalizations for otitis media by *pseudomonas, pneumonia, influenza*, and other acute respiratory tract infections of hospitalized patients differed by age and gender. The male have hospitalization rate ratio (HRR) for admission due to a respiratory tract infection more than female. \cite{4}

In humans, *P. aeruginosa* is the most commonly isolated bacterial pathogen in adults and children with a clinical history of chronic, suppurative otitis media, and a major cause of otitis media in neonates\cite{11}. This is in contrast to studies which demonstrated that *P. aeruginosa* is not a normal inhabitant of the external auditory canal, having been recovered from that site in only 1% of 1,377 healthy volunteers\cite{12}.

<table>
<thead>
<tr>
<th>Age</th>
<th>Male</th>
<th>%</th>
<th>Female</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>2</td>
<td>18</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>10-15</td>
<td>5</td>
<td>45</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>15-20</td>
<td>1</td>
<td>10</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>20-25</td>
<td>3</td>
<td>27</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>27</td>
<td>11</td>
<td>30</td>
</tr>
</tbody>
</table>

Table-1: Distribution of otitis media patients according to different ages and sex.

Isolation and Identification of bacteria:

Twenty Clinical swab specimens taken from middle ear infection patients were cultured on cetramide agar plates, ten isolates which may be belonged to be *Pseudomonas* were further identified according to morphological characteristic and biochemical tests.

There have been reported cases of clinical inner ear disease in mice affecting the vestibular apparatus, attributed to natural *P. aeruginosa* infection\cite{13,14}. These were characterized clinically by either circling\cite{15} or rolling\cite{16}. Similar signs of spinning/circling had also been experimentally produced by intravenous inoculation of mice with *P. aeruginosa*\cite{17}.
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For the former, colonies of each isolate was plated on nutrient agar showed different morphological characteristics of mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color, have fruity odor, all of them were pyocynine producers, and the shape of the colony appears like a fried egg shape, these result are reasonable with the result demonstrated by [7, 18]. Microscopically examination of each isolate showed that they were all motile, non –spore forming, gram negative and rod shape. Furthermore, six isolates that were suspected to be belongs to Pseudomonas sp. were subjected to a number of biochemical tests. Results indicated in table-2 showed that these isolates gave a positive result for oxidase and catalase and were able to growth on cetramide medium which indicated that these isolates belong to Pseudomonas sp. [19] indicated that only Pseudomonas species were able to grow on cetramide medium. Glucose hydrolysis test was also performed to characterize P. aeruginosa from other species. On the other hand, these isolates differ in growth at 42°C and 4°C, five isolates (P1, P2, P3, P4 and P5) were able to grow at 41°C but they can not grow at 4°C. It was found that five isolates were belonging to P. aeruginosa. Our results were in agreement to result obtained by [19].

<table>
<thead>
<tr>
<th>Tests</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony color</td>
<td>Green</td>
</tr>
<tr>
<td>Growth on cetramide medium</td>
<td>+ve</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Gram stain</td>
<td>-ve</td>
</tr>
<tr>
<td>Catalaseoduction Pr</td>
<td>+ve</td>
</tr>
<tr>
<td>Oxidase production</td>
<td>+ve</td>
</tr>
<tr>
<td>Growth at 4° C</td>
<td>-ve</td>
</tr>
<tr>
<td>Growth at 42 ° C</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Table-2: Some biochemical tests and morphological examination to identify Pseudomonas Sp.

Antibiotic sensitivity test:

The development of antibiotic resistance is considered a major therapeutic problem that can be explained by some hypothesis such as, the influence of excessive and/or inappropriate antibiotic use [20].

Standard disk diffusion test has been performed for detection of susceptibility of pathogenic P. aeruginosa five isolates for several antibiotic disks, decision for considering an isolate as resistant or sensitive was taken in comparison of the diameter of inhibition zone with that of standard value of [21]. As shown in table-3.
Table-3: Antibiogram of *P. aeruginosa* isolates isolated from otitis patients

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>CTX (10) µg</th>
<th>AMX (10) µg</th>
<th>C (30) µg</th>
<th>CIP (5) µg</th>
<th>FOX (30) µg</th>
<th>TE (30) µg</th>
<th>P (10) µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S(25m m)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>P2</td>
<td>S(25mm)</td>
<td>R</td>
<td>R</td>
<td>S(20m m)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>P3</td>
<td>S(25mm)</td>
<td>R</td>
<td>S(15m m)</td>
<td>S(22m m)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>P4</td>
<td>R</td>
<td>S(15mm)</td>
<td>R</td>
<td>S(30m m)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>P5</td>
<td>S(12mm)</td>
<td>R</td>
<td>S(23m m)</td>
<td>S(23m m)</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

S=Sensitive, R=Resistant, CTX=Cefotaxime, AMX=Amoxicillin, C=Chloramphenicol, CIP=Ciprofloxacin, FOX=cefoxitin, TE=tetracycline and P=Penicillin

Result shown in table-3 indicated that resistance to antibiotics was widely distributed among isolates, however, they varies according to nature of the isolate and kind of antibiotics when all isolates (P1, P2, P3, P4 and P5) showed resistance for (Tetracyclin, Pencilline, Cefoxitin) and to (Amoxicilin) except P4 also three isolates showed resistance to Chloraemphicol (P1, P2 andP4) which were sensitive to it except(P3, P5) while all isolates showed sensitivity to Ciprofloxacin and cefotaxime except (P1, P4) which were resistance to the former one.

From these results it was concluded that *P. aeruginosa* is probably has the ability to produce more than one enzyme among them β-lactamases. Bacterial resistance to beta-lactam antibiotics can be achieved by any of three strategies: the production of beta-lactam-hydrolyzing beta-lactamase enzymes, the utilization of beta-lactam-insensitive cell wall transpeptidases, and the active expulsion of beta-lactam molecules from Gram-negative cells by way of efflux pumps. The greater sensitivity observed with Ciprofloxacin which gives the largest zone of inhibition compared with other antibiotics. This result was in agreement with result obtained by who found that ciprofloxacin was effective in the treatment of the virulent gram negative bacteria including *P. aeruginosa*.

The mechanism of resistance for ciprofloxacine included effective suction pump of the antibiotic from inside to outside to escape its effect and prevent the accumulation of antibiotic inside bacterial cell. From this result of table-3 one could conclude that ciprofloxacin remain the first choice when all isolates were sensitive to it.

The percentage of *P. aeruginosa* resistance isolates to each antibiotics were shown in figure-2, when all isolates were resistance to pencilline,
tetracycline, Cefotaxtin (100%) and (60%) of them showed resistance to cefotaxime and (40%) to cloraemphenicol and (20%) to amoxicillin while all isolates were sensitive to ciprofloxacin.

![Percentage resistant of P. aeruginosa isolates](image)

**Figure-1: Percentage resistant of P. aeruginosa isolates**

The number of multiple antibiotic resistance strains has been increasing since resistance is mainly mediated by R- plasmids which determined beta-lactamase in Gram negative bacilli [20] all the R-plasmids carried the markers of resistance to cloraemphenicol, tetracycline, ampicillin, gentamycin and streptomycin [21].

In the case of ciprofloxacin none of P. aeruginosa isolates were resistance to it because ciprofloxacin is a fluoroquinone antibiotic with broad spectrum bacterial activity. Ciprofloxacin inhibit bacterial DNA gyrase, so preventing the supercoliong of DNA, a process that is necessary for compacting chromosomes into the bacterial cell [22,25].

**Minimal Inhibitory Concentration (MIC) Test:**

*P. earuginosa* infections are more difficult to treat due to the organisms high intrinsic resistance to many antimicrobial agents, this resistance is partly due to its relatively low outer membrane permeability [26]. However there are other mechanisms that included decreasing the passage into or increasing the efflux of drug from bacterial cell and modification of the target site.

Tow isolates were selected due to multiple antibiotic resistance, therapy, these isolates were selected in order to evaluate bacteria susceptibility to antibiotic that revealed earlier by disk diffusion test when examined against antibiotics. MIC was defined as lowest concentration of antibiotic in microgram per milliliter that prevents the in *vitro* growth of bacteria. The MIC test was performed to determine their minimal inhibition concentration as in table-4, both P1and P2 showed high-level of resistance to Cefotaxime and cloraemphenicol with MIC (128µg/ml), followed by (64µg/ml) to ampicillin while P2 was (128
µg/ml), as well as, the amoxicillin showed the lowest MIC (16 µg/ml) for P1 and (64 µg/ml) to P2.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>C (µg/ml)</th>
<th>AMX (µg/ml)</th>
<th>AM (µg/ml)</th>
<th>CTX (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>128</td>
<td>16</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>P2</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td>128</td>
</tr>
</tbody>
</table>

Table-4: The MIC value of some antibiotics for P. aeruginosa (P1, P2) isolates.
C=Chloramphenicol, AMX=Amoxicillin, AM=Ampicillin and CTX=Cefotaxime.

P. aeruginosa has an outer membrane with a low permeability and most antimicrobial agents were not easily diffused through. Active efflux to be especially effective mechanisms of antibiotic resistance in this bacterium[27] indicated that MIC values are greatly affected by environmental conditions including the pressure of CO2 concentration causing acidification of the test media.

Antibacterial effect of garlic on Pseudomonas aeruginosa:
The phytoconstituents of garlic have longed been known and its antimicrobial properties have been widely reported [28]. The antimicrobial activities of plant extracts including garlic have been linked to the presence of some bioactive compounds. These secondary metabolites also serve to protect the plants themselves against bacterial, fungal and viral infections [29]. These bioactive compounds are known to work synergistically to produce various effects on the human and animal subjects [30]. However, most reports on the activity of garlic have focused mainly on the commensally microflora and community acquired infections, while informations on its activity against hospital based pathogens is scanty.

The pH of each of the garlic solution was 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8. The activity of the garlic was tested in these different pH levels and has no pH effect on the garlic activity been observed. This was similar to the observation of[31].

The MIC of garlic extract on Pseudomonas aeruginosa at different incubation periods was determined. Table-5 showed that after 24hrsMIC of extract was greater than 8µg/ml for P1 isolates and 16µg/ml for P2 isolates which is almost similar with the work of [31]. The lower concentration of garlic had no antibacterial effect in this work, however; it may effective as [31]. This is may be due the species difference or the garlic difference in different biologic condition.
Table (5) The MIC value for Garlic extract on P. aeruginosa (P1, P2) after 24hrs.

<table>
<thead>
<tr>
<th>No .of isolate</th>
<th>Concentration of garlic µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>P1</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
</tr>
</tbody>
</table>

+=Indicates growth of P.aeruginosa and – = no growth

Table (6): The MIC value for Garlic extract on P. aeruginosa (P1, P2) after 72hrs.

<table>
<thead>
<tr>
<th>No .of isolate</th>
<th>Concentration of garlic µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>P1</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
</tr>
</tbody>
</table>

+=Indicates growth of P.aeruginosa and – = no growth

In the table (6) the MIC of garlic extract on P. aeruginosa was determined after 72rs and it was found that at concentrations (32, 64 and 128 µg/ml) were more effective in inhibition growth of (P1, P2) while in (8 µg/ml) that indicated heavy growth of P. aeruginosa.

It has shown that dilute solutions of garlic can completely inhibit the growth of P.aeruginosa at the concentration of more than 16 µg/ml. This could be due to the action of biological active ingredient of allicin exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action. From our study it was concluded that Psudomonas aeruginosa were highly prevalence among teenager age of otitis patients and High prevalence of antibiotic resistance was observed among Psudomonas aeruginosa isolates especially to penicillin cefotaxin and tetracycline and it was found that garlic extract has considerable inhibitory effects against the tested P.aeruginosa isolates

References:


Evaluate the antibacterial effect of Garlic (*Allium sativum*) and antimicrobial susceptibility on *Pseudomonas aeruginosa* isolated from otitis media

Maysaa Ch. Al-Yas  
*Biotechnology Department, College of Science, Al-Nahrain University*

Abstract:

Twenty swab samples were taken from patients suffering from otitis infection; these samples were cultured on different agar media (blood agar base, macConky and cetramide). Several microscopical, morphological and biochemical tests were performed and results showed that 50% of isolated colony were belonged to *Psudomonas aeruginosa*. In addition it was found that males were more susceptible to infection with otitis than female especially at teenager aged.
Five isolates were subject to sensitivity test against several antibiotic groups. Result showed that all five isolates were resistance to pencilline, tetracycline, Cefotaxin (100%) and (60%) of them showed resistance to cefotaxime and (40%) to chloroemphecol and (20%) to amoxicillin while all isolates were sensitive to ciprofloxacin. The minimum inhibitory concentration (MIC) test was performed for two multiple drugs resistance isolates (P1 and P2) result declared that both P1and P2 showed high-level of resistance to Cefotaxime and chloroemphenicol with MIC (128µg/ml), followed by (64µg/ml) to ampicillin while P2 was (128 µg/ml), as well as, the amoxicillin showed the lowest MIC (16 µg/ml) for P1 and (64 µg/ml) to P2.

No pH effect was observed on the activity of garlic when antibacterial effect of garlic extract against *Pseudomonas aeruginosa* was investigated at different PH. The MIC of garlic extract on *Pseudomonas aeruginosa* at different incubation periods was determined. Results showed that after 24hrs MIC of extract was greater than 8µg/ml for P1 isolate and 16µg/ml for P2 isolate. While, after 72rs it was found that the MIC at concentrations (32, 64 and 128 µg/ml) were more effective in inhibition growth of (P1, P2) while in (8 µg/ml) that indicated heavy growth of *P. aeruginosa*

**Introduction:**

Otitis media (Latin for "Middle otitis") is inflammation of the middle ear, or middle ear infection. Otitis media occurs in the area between the ear drum (the end of the outer ear) and the inner ear, including a duct known as the Eustachian tube.\[1\]

Middle ear infection is mainly a problem in children, although it also occurs in adults. \[2\] declared that otitis media is one of most common disease of children which is the leading cause hearing loss in children and the most frequent indication for antimicrobial or therapy in children.

*Pseudomonas* spp. were the most commonly identified etiologic agents causing dermatitis, conjunctivitis, or otitis. In humans, *P. aeruginosa* is the second most frequent gram-negative nosocomial pathogen in hospitals.\[3\] *P.aeruginosa* is frequently found in the normalear and is the predominant bacterial pathogen in some cases of external otitis . The bacterium can cause a more serious ear infection in elderly patients, possibly leading to hearing problems, facial paralysis, or even death. Ear infection of *P. aeruginosa* can cause infections in the external ear canal-- so-called swimmer's ear"-- that usually disappear without treatment.\[4\]

There is extensive literature on the antibacterial effects of fresh garlic juice, aqueous and alcoholic extracts, lyophilized powders, steam distilled oil and other commercial preparations of garlic. It is a broad spectrum antibiotic, killing a wide variety of bacteria. Many pharmaceutical antibiotics kill only a narrow range of these germs while garlic has the broadest spectrum of any antimicrobial substance that otitis know due to antibacterial, antifungal,
antiparasitic, antiprotozoan and antiviral. This property belongs to the garlic constituent allicin, which is released when cutting garlic clove. [5] Moreover, garlic extracts exhibited activity against both gram negative (E. coli, Salmonella sp. and Citrobacter, enterobacter, Pseudomona and Klebsiella) and gram positive (S. aureus, S. pneumonia Group A Streptococcus and Bacillus anthrcis) all of which are cause of morbidity worldwide. [6] The present study tested an aqueous extract of dried garlic in vitro for its antibacterial activity against Pseudomonas aeruginosa isolates isolated from otitis patients with determination their susceptibility to antimicrobial agents.

Materials and Methods:
Bacterial isolation:

Twenty swab samples were obtained from Al-Yarmook Teaching Hospital in Baghdad from the periods (1/9/2009-1/10/2009) from patients suffering from otitis disease. Specimens were obtained by sterile cotton swab these were processed for direct examination and cultivation on blood agar media, MacConkeys agar and cetramide agar media (Banagalore, India) and were identified initially as Pesudomonas species according to their morphological, physiological and biochemical properties as indicated by [7,8] depending on Gram stain, colony shape, oxidase, catalase, growth on cetramide medium, production of pyocynine, growth at 4 and 42 °C.

Antibiotic susceptibility

The use of antimicrobial sensitivity test is essential for the selection of an appropriate drug for treatment of otitis infection. The disc diffusion method was used in this study against 5 isolates of Pseudomonas aeruginosa depending on the Kirby-Bauer diffusion method [9]. Up to 7 different groups of discs of the available antimicrobial agents were used in this study.

Minimum inhibitory concentration:

Broth microdilution method was performed, in this experiment twofold dilutions of antibiotics were done in broth media and broth was inoculated with 105 CFU/ml of the tested organisms. After incubation for 18-24 hrs, MIC was described as concentration in which no visible growth was observed. [8]

Preparation of garlic extract:

The garlic bulbs were washed thoroughly under tap running water aseptically cut into small pieces with a knife and then kept in the shade for 1-3 days at 32-35°C. The semi-dried pieces were then crushed using pestle and mortar, and left to dry in the shade at room temperature for further two days. The dried garlic materials were further ground to powdery form with a Kenwood blender. Two hundred gram (200.0 g) of garlic powder was extracted with 500 ml of solvents distilled water, for 24 h by using Soxhlet apparatus. The extract were concentrated using a rotary evaporator at 40°C. Clearly prepared garlic powder was thoroughly mixed with distilled water and the concentration was determined with varying amounts of crude preparation of garlic to give the final
concentration of 8, 16, 32, 64 and 128 mg/ml of brain heart infusion broth media and the final volume of 10 ml.\textsuperscript{10}

\section*{Results and Discussion:}

\subsection*{Isolation of \textit{Pseudomonas} Species:}

Twenty swab samples were collected from patients suffering from middle ear infection (otitis) (male and female) from Al-Yarmook teaching Hospital belongs to variety ages from (5-25) years. Results in table (3) showed that males were more susceptible to infection with otitis than female especially at teenager aged the reason for that is male have hospitalization rate ratio (HRR) for admission due to a respiratory tract infection more than female. Hospitalizations for otitis media by \textit{pseudomonas, pneumonia, influenza}, and other acute respiratory tract infections of hospitalized patients differed by age and gender. The male have hospitalization rate ratio (HRR) for admission due to a respiratory tract infection more than female.\textsuperscript{14}

In humans, \textit{P. aeruginosa} is the most commonly isolated bacterial pathogen in adults and children with a clinical history of chronic, suppurative otitis media, and a major cause of otitis media in neonates\textsuperscript{11}. This is in contrast to studies which demonstrated that \textit{P. aeruginosa} is not a normal inhabitant of the external auditory canal, having been recovered from that site in only 1\% of 1,377 healthy volunteers\textsuperscript{12}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Age} & \textbf{Male} & \textbf{Female} & \textbf{Male} & \textbf{Female} \\
\hline
5-10 & 2 & 18 & 3 & 30 \\
10-15 & 5 & 45 & 1 & 18 \\
15-20 & 1 & 10 & 2 & 22 \\
20-25 & 3 & 27 & 3 & 30 \\
\textbf{Total} & 11 & 9 & 30 & 1 \\
\hline
\end{tabular}
\caption{Distribution of otitis media patients according to different ages and sex.}
\end{table}

\subsection*{Isolation and Identification of bacteria:}

Twenty Clinical swab specimens taken from middle ear infection patients were cultured on cetramide agar plates, ten isolates which may be belonged to be \textit{Pseudomonas} were further identified according to morphological characteristic and biochemical tests.

There have been reported cases of clinical inner ear disease in mice affecting the vestibular apparatus, attributed to natural \textit{P. aeruginosa} infection\textsuperscript{13,14}. These were characterized clinically by either circling\textsuperscript{15} or rolling\textsuperscript{16}. Similar signs of spinning/circling had also been experimentally produced by intravenous inoculation of mice with \textit{P. aeruginosa}\textsuperscript{17}.
For the former, colonies of each isolate was plated on nutrient agar showed different morphological characteristics of mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color, have fruity odor, all of them were pyocynine producers, and the shape of the colony appears like a fried egg shape, these result are reasonable with the result demonstrated by [7, 18]. Microscopically examination of each isolate showed that they were all motile, non –spore forming, gram negative and rod shape. Furthermore, six isolates that were suspected to be belongs to *Pseudomonas* sp. were subjected to a number of biochemical tests. Results indicated in table-2 showed that these isolates gave a positive result for oxidase and catalase and were able to growth on cetramide medium which indicated that these isolates belong to *Pseudomonas* sp. [19] indicated that only *Pseudomonas* species were able to grow on cetramide medium. Glucose hydrolysis test was also performed to characterize *P. aeruginosa* from other species. On the other hand, these isolates differ in growth at 42°C and 4°C, five isolates (P1, P2, P3, P4 and P5) were able to grow at 41°C but they can not grow at 4°C. It was found that five isolates were belonging to *P. aeruginosa*. Our results were in agreement to result obtained by [19].

<table>
<thead>
<tr>
<th>Tests</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony color</td>
<td>Green</td>
</tr>
<tr>
<td>Growth on cetramide medium</td>
<td>+ve</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Gram stain</td>
<td>-ve</td>
</tr>
<tr>
<td>Catalaseoduction Pr</td>
<td>+ve</td>
</tr>
<tr>
<td>Oxidase production</td>
<td>+ve</td>
</tr>
<tr>
<td>Growth at 40°C</td>
<td>-ve</td>
</tr>
<tr>
<td>Growth at 42 °C</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Table-2: Some biochemical tests and morphological examination to identify *Pseudomonas* Sp.

**Antibiotic sensitivity test:**

The development of antibiotic resistance is considered a major therapeutic problem that can be explained by some hypothesis such as, the influence of excessive and/or inappropriate antibiotic use [20].

Standard disk diffusion test has been performed for detection of susceptibility of pathogenic *P. aeruginosa* five isolates for several antibiotic disks, decision for considering an isolate as resistant or sensitive was taken in comparison of the diameter of inhibition zone with that of standard value of [21]. As shown in table-3.
Result shown in table-3 indicated that resistance to antibiotics was widely distributed among isolates, however, they varies according to nature of the isolate and kind of antibiotics when all isolates (P1, P2, P3, P4 and P5) showed resistance for (Tetracyclin, Pencilline, Cefoxitin) and to (Amoxicilline) except P4 also three isolates showed resistance to Chloramphicol (P1, P2 and P4) which were sensitive to it except(P3, P5) while all isolates showed sensitivity to Ciprofloxacin and cefotaxime except (P1, P4) which were resistance to the former one.

From these results it was concluded that *P. aeruginosa* is probably has the ability to produce more than one enzyme among them β-lactamases. Bacterial resistance to beta-lactam antibiotics can be achieved by any of three strategies: the production of beta-lactam-hydrolyzing beta-lactamase enzymes, the utilization of beta-lactam-insensitive cell wall transpeptidases, and the active expulsion of beta-lactam molecules from Gram-negative cells by way of efflux pumps. The greater sensitivity observed with Ciprofloxacin which gives the largest zone of inhibition compared with other antibiotics. This result was in agreement with result obtained by who found that ciprofloxacin was effective in the treatment of the virulent gram negative bacteria including *P.aeruginosa*.

The mechanism of resistance for ciprofloxacine included effective suction pump of the antibiotic from inside to outside to escape its effect and prevent the accumulation of antibiotic inside bacterial cell. From this result of table-3 one could conclude that ciprofloxacin remain the first choice when all isolates were sensitive to it.

The percentage of *P. aeruginosa* resistance isolates to each antibiotics were shown in figure-2, when all isolates were resistance to pencilline,
tetracycline, Cefotaxin (100%) and (60%) of them showed resistance to cefotaxime and (40%) to chloramphenicol and (20%) to amoxicillin while all isolates were sensitive to ciprofloxacin.

![Image](https://via.placeholder.com/150)

**Figure-1: Percentage resistant of P. aeruginosa isolates**

The number of multiple antibiotic resistance strains has been increasing since resistance is mainly mediated by R- plasmids which determined beta-lactamase in Gram negative bacilli[20] all the R-plasmids carried the markers of resistance to chloramphenicol, tetracycline, ampicillin, gentamycin and streptomycin[21].

In the case of ciprofloxacin none of the strains were resistance to it because ciprofloxacin is a fluoroquinone antibiotic with broad spectrum bacterial activity. Ciprofloxacin inhibit bacterial DNA gyrase, so preventing the supercoiling of DNA, a process that is necessary for compacting chromosomes into the bacterial cell [22,25].

**Minimal Inhibitory Concentration (MIC) Test:**

*P. aeruginosa* infections are more difficult to treat due to the organisms high intrinsic resistance to many antimicrobial agents, this resistance is partly due to its relatively low outer membrane permeability [26]. However there are other mechanisms that included decreasing the passage into or increasing the efflux of drug from bacterial cell and modification of the target site.

Two isolates were selected due to multiple antibiotic resistance, therapy, these isolates were selected in order to evaluate bacteria susceptibility to antibiotic that revealed earlier by disk diffusion test when examined against antibiotics. MIC was defined as lowest concentration of antibiotic in microgram per milliliter that prevents the in vitro growth of bacteria. The MIC test was performed to determine their minimal inhibition concentration as in table-4, both P1 and P2 showed high-level of resistance to Cefotaxime and chloramphenicol with MIC (128µg/ml), followed by (64µg/ml) to ampicillin while P2 was (128
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<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>C  µg/ml</th>
<th>AMX µg/ml</th>
<th>AM  µg/ml</th>
<th>CTX µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>128</td>
<td>16</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>P2</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td>128</td>
</tr>
</tbody>
</table>

Table-4: The MIC value of some antibiotics for *P. aeruginosa* (P1, P2) isolates.
C=Chloramphenicol, AMX=Amoxicillin, AM=Ampicillin and CTX=Cefotaxime.

*P. aeruginosa* has an outer membrane with a low permeability and most antimicrobial agents were not easily diffused through. Active efflux to be especially effective mechanisms of antibiotic resistance in this bacterium indicated that MIC values are greatly affected by environmental conditions including the pressure of CO2 concentration causing acidification of the test media.

**Antibacterial effect of garlic on *Pseudomonas aeruginosa*:**

The phytoconstituents of garlic have longed been known and its antimicrobial properties have been widely reported. The antimicrobial activities of plant extracts including garlic have been linked to the presence of some bioactive compounds. These secondary metabolites also serve to protect the plants themselves against bacterial, fungal and viral infections. These bioactive compounds are known to work synergistically to produce various effects on the human and animal subjects. However, most reports on the activity of garlic have focused mainly on the commensally microflora and community acquired infections, while informations on its activity against hospital based pathogens is scanty.

The pH of each of the garlic solution was 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8. The activity of the garlic was tested in these different pH levels and has no pH effect on the garlic activity been observed. This was similar to the observation of.

The MIC of garlic extract on *Pseudomonas aeruginosa* at different incubation periods was determined. Table-5 showed that after 24hrsMIC of extract was greater than 8µg/ml for P1 isolates and 16µg/ml for P2 isolates which is almost similar with the work of. The lower concentration of garlic had no antibacterial effect in this work, however; it may effective as. This is may be due the species difference or the garlic difference in different biologic condition.
Table (5) The MIC value for Garlic extract on *P. aeruginosa* (P1, P2) after 24hrs.

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<th>No .of isolate</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 µg/ml</td>
</tr>
<tr>
<td>P1</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>+</td>
</tr>
</tbody>
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+=Indicates growth of *P. aeruginosa* and -= no growth

Table (6): The MIC value for Garlic extract on *P. aeruginosa* (P1, P2) after 72hrs.

<table>
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In the table (6) the MIC of garlic extract on *P. aeruginosa* was determined after 72rs and it was found that at concentrations (32, 64 and 128 µg/ml) were more effective in inhibition growth of (P1, P2) while in (8 µg/ml) that indicated heavy growth of *P. aeruginosa*.

It has shown that dilute solutions of garlic can completely inhibit the growth of *P. aeruginosa* at the concentration of more than 16 µg/ml. This could be due to the action of biological active ingredient of allicin exhibits its antimicrobial activity mainly by immediate and total inhibition of RNA synthesis, although DNA and protein syntheses are also partially inhibited, suggesting that RNA is the primary target of allicin action. From our study it was concluded that *Psudomonas aeruginosa* were highly prevalence among teenager age of otitis patients and High prevalence of antibiotic resistance was observed among *Psudomonas aeruginosa* isolates especially to penicillin cefotaxin and tetracycline and it was found that garlic extract has considerable inhibitory effects against the tested *P.aeruginosa* isolates.

References:


The comparison study of Fluoride kinetics in saliva after the use of chewing gums containing different fluoride compounds In Iraqi subjects

Khalid Nassif Jassim
College of Dentistry, University of Al Mustansiryia

Abstract:
There is a relationship between the use of fluoride, the reduction of dental caries and the increase of dental fluorosis. The purpose of this study was to analyze the fluoride kinetics in saliva after using two commercially available chewing gums with fluoride, which contains (3.38) mg of fluoride as marketed Sodium monofluorophosphate (NaMFP) and sodium fluoride (NaF) which are the two most common sources of fluoride used in currently fluoride dentifrices.

Fifteen 7-9 year-old volunteers were instructed to chew the fluoridated gums. Total saliva was collected, at 0, 3, 6, 9 and 15 minutes after 3 minutes...
starting chewing. Salivary fluoride was analyzed with a fluoride-specific electrode (201 Fluoride E. HANNA instruments) after acid hydrolysis.

The fluoride amount in the saliva samples after the use of chewing gum which contains Sodium monofluorophosphate (NaMFP) was 0.4 mg which higher than after the use of chewing gum contains sodium fluoride (NaF) which was 0.041 mg in all experimental periods,

The high fluoride presence in saliva after the use of chewing gum of (NaMFP) is significant to prevent dental caries and this should be evaluated in clinical researches. On the other hand, children at an age of risk for dental fluorosis should avoid the use of this kind of chewing gums.

**Key words:** Chewing fluoride gum, fluoride kinetics, saliva, Sodium monofluorophosphate, sodium fluoride, fluorosis

**Introduction:**

Dental caries is a multifactorial disease and one of the main public health problems. As a result of several studies carried out to understand the dental caries process as well as its risk factors, dental treatment emphasis has been moved from corrective to preventive methods. Fluoride is an extensively proven effective agent for the control of dental caries. Its cariostatic effect is related to its presence in the aqueous phase of the apatite crystals [1, 2, 3], which inhibits demineralization and activates remineralization [4].

There are many studies supporting the frequent and repetitive use of low concentration and self-applied fluoride agents [5, 6], stimulating the search for alternative devices to apply it in the mouth, such as professional products, at high fluoride concentrations (solutions, gels, pastes and varnishes) or through home care products, like toothpastes, mouth washes, fluoridated mucoadhesive tablets, and, more recently, fluoridated chewing gums [7, 8].

The use of chewing gum increases the salivary flow, which helps to clean the oral cavity [9]. Chewing gums have been also introduced as useful vehicles for fluoride, calcium, phosphate and chlorhexidine delivery [10]. Fluoride-containing chewing gums increase salivary and dental plaque pH, calcium and phosphate concentration and also act on enamel remineralization [11].

The chewing gums of (NaMFP) and (NaF) were recently introduced in the marketplace as an additional agent to prevent dental caries. Each piece of the product contains 3.38 mg of fluoride as monofluorophosphate or sodium fluoride, despite the possibility of helping to prevent dental caries, a concern arises about its contribution as an additional source of fluoride intake when consumed by children in the age of risk for dental fluorosis.

Dental fluorosis is a health condition caused by a child receiving too much fluoride during tooth development. The critical period of exposure is between 1 and 4 years old is more risky than children over 8 years old [12]. In its mild form, which is the most common, fluorosis appears as tiny white streaks
or specks that are often unnoticeable. In its severe form it is characterized by black and brown stains, as well as cracking and pitting of the teeth\cite{13}.

The severity of dental fluorosis depends on the amount of fluoride exposure, the age of the child, individual response, and nutritional and other factors \cite{12}. Although water fluoridation can cause fluorosis, most of this is mild (0.7-1.2 mg of fluoride/L) and not usually of aesthetic concern\cite{14}. Severe cases can be caused by exposure to water that is naturally fluoridated to levels well above the recommended levels, or by exposure to other fluoride sources such as brick tea or pollution from high fluoride coal\cite{15}.

According to the centers for diseases control, 32% of American children now have some form of dental fluorosis, with 2 to 4% of the children having the moderate to severe stage (CDC 2005 USA).

Thus, the aim of the present study was to analyze the amount of fluoride released in saliva after chewing the gums of (NaMFP) and (NaF) in Iraqi subjects.

\begin{center}
\[
\text{Chemical Structure of Sodium monofluorophosphate}
\]
\end{center}

\begin{center}
\[
\text{Na}^+ \text{O} \equiv \text{P} \equiv \text{O}^- \text{Na}^+ \quad \text{F}
\]
\end{center}

\textbf{Materials and Methods:}

\textbf{Experimental design:}

The study was carried out with fifteen 7-12 year-old children. All of them had good general health; the children chewed the fluoridated gum, each piece containing 4.2 mg of fluoride as monofluorophosphate or sodium fluoride.

The saliva was collected in cooled plastic containers at (0, 3, 6, 9, and 15 min) after 3 minutes starting chewing gum. During this period, the volunteers remained seated and were not allowed to have any food or drink. The samples obtained were stored in freeze at -5°C until fluoride analysis.

\textbf{Fluoride analysis:}

\textbf{Acid hydrolysis:}

The acid hydrolysis of fluoride was done because the samples of chewing gums contain fluoride as monofluorophosphate and sodium fluoride which form complex with some of saliva components. The method used was adapted from the method proposed by Cury\cite{16} modified by Orth \textit{et al.}\cite{17} for the analysis of monofluorophosphate and sodium fluoride in saliva after using a dentifrice containing both these fluoridated compounds. To 0.25 ml of each saliva sample, 0.25 ml of 2 mol L\textsuperscript{-1} hydrochloric acid (Analytical reagent, Gainland chemical company, UK) was added, and the samples were kept for 1 hour at 45°C under
agitation in water bath. Then, neutralization was accomplished with 0.5 ml of 1 mol L\(^{-1}\) sodium hydroxide (Analytical reagent, Gainland chemical company, UK).

Fluoride measurement

Fluoride was analyzed by the direct method, using a fluoride specific electrode (201 Fluoride E. HANNA instrument, China) and an ion analyzer. Prior to the samples analysis, a set of standards (ranging between 0.025-3.2 ppm F) was prepared in triplicate, using serial dilution from a 100 ppm NaF stock solution (E.Merck, Darmstadt, Germany). The millivoltage potentials were converted to µg F using a standard curve.

![Figure-1: The kinetic released of fluoride (mg/g) in saliva from chewing gum of NaMFP and NaF respectively at different periods (min).](image)

Result:

Figure-2 shows the mean total fluoride released (mg) with time. The comparisons showed that the chewing gum which contains (NaMFP) released significantly higher amounts of fluoride when compared to chewing gum which contains (NaF), up to the 15 min collection time. The amount released decreased with time.

Table -1 represents the total amount of fluoride released in saliva (mg), during the whole experiment, for the two gums, a significantly higher amount of fluoride was released when chewing gum which contains (NaMFP ) was chewed (0.4) mg of fluoride when compared to chewing gum which contains NaF (0.041) mg of fluoride.
Table 1: The concentration of fluoride which released in saliva from fluoridated chewing gum which indicated a significant increase (P<0.05) in release from NaMFP compared with NaF.

<table>
<thead>
<tr>
<th>Collection period (min)</th>
<th>Fluoride conc. Released from NaMFP chewing gum (mg)</th>
<th>Fluoride conc. Released from NaF chewing gum (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.26</td>
<td>0.025</td>
</tr>
<tr>
<td>3</td>
<td>0.06</td>
<td>0.007</td>
</tr>
<tr>
<td>6</td>
<td>0.004</td>
<td>0.005</td>
</tr>
<tr>
<td>9</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>15</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>0.4</td>
<td>0.041</td>
</tr>
</tbody>
</table>

The contribution as a percentage, of one piece of each chewing gum to the maximum daily recommended fluoride intake (0.07 mg of fluoride/kg of body weight)\(^{18}\). For 1 to 7 years old children a single tablet of chewing gum of NaMFP represent 57% and 23.8% and for NaF chewing gum represents 5.85% and 2.56% of the maximum daily fluoride ingestion recommended for children aged 1 and 7 years old, respectively.

Discussion:

It is known that the frequent and repeated use of low fluoride concentration products, which promote low and constant salivary fluoride levels, is the most efficient way to prevent dental caries\(^{11,19,10}\). Levels between 1 and 10 ppm of fluoride reduce the enamel solubility and increase the remineralization rate, facilitating the precipitation of minerals on the enamel surface\(^{11,19}\).

Sjögren et al\(^{10}\) (1997) found the greatest fluoride values in saliva between 5 and 10 minutes after the use of a fluoridated chewing gum. In the subsequent periods, the fluoride concentration in saliva decreased gradually. In this study, the fluoride release was significantly higher at 0, 3 min compared to 6, 9 and 15 min when the fluoridated gum was chewed. Although Sjögren et al\(^{10}\) (1997) also suggest that the salivary flow stimulation may have a negative effect on the fluoride retention in the mouth, Bruun et al\(^{13}\). (1982) demonstrated that a single chewing gum tablet with 0.5 mg of fluoride can maintain high fluoride levels in saliva for at least 60 min.

Lamb et al\(^{11}\). (1993) sustained in their study that chewing a fluoridated gum with only 0.1 mg of fluoride five times a day favors the remineralization of initial dental caries lesions, and that this frequency could maintain high fluoride levels in saliva during most part of the day.
Silva et al.[20]. (2003) evaluated the effect of two commercially available chewing gums with fluoride on the cariogenic micro biota of saliva and dental plaque. The gum Fluorite showed a faster pH recovery and a F release to saliva after up to 30 minutes.

The potential anticariogenic effect of chewing gums containing fluoride has been proposed by Lamb et al. [11] (1993) and Sjögren et al.[10]. (1997). However, there are no data regarding the fluorosis risk that this type of product may promote. It has been suggested that the incidence and severity of dental fluorosis have become greater in the last decade in both optimally fluoridated and non-fluoridated areas in many countries, as well as in Brazil[21, 22, 23, 24, 25].

This has been attributed to an increase in the fluoride level of foods and beverages through processing with fluoridated water, inadvertent ingestion of fluoride toothpaste, and the inappropriate use of dietary supplements[7].

Considering that the highest risk factor for the development of dental fluorosis is the total amount of fluoride ingested, and that nowadays there are several available sources, the chewing gum of NaMFP can cause concern for children at the age of risk for dental fluorosis, which comprises 1 to 7 years of age.

One tablet of NaMFP chewing gum could represent 57%, (NaMFP is easily soluble in water with strong hygroscopy, its solubility is 42gm\100 gm water at 25°C) while one tablet of NaF chewing gum could represent 5.85% only of the maximum daily fluoride ingestion (the relative constant solubility rate of NaF makes it an ideal source for the fluoride ion in treatment of municipal water supplies ,solubility 0.76g\100gm water at 25°C). This maximum daily ingestion was calculated, based on the literature, as being 0.07 mg of fluoride/kg of body weight[18] and considering the use of a single tablet. Unfortunately, there is no data available on the consumption of chewing gum by children. However, it is possible that children use more than one tablet per day, which increases the risk of dental fluorosis development.

Hattab et al. [26] (1989) demonstrated a mild increase of fluoride plasma levels after the use of a fluoridated chewing gum and concluded that this product offers a minimal risk of adverse effects. However, this study was carried out in fluoride deficient areas and with adult subjects, using a chewing gum with smaller amounts of fluoride (0.113 mg) when compared to that used by the present study (approximately 3.38 mg, according to the manufacturer).

Therefore, other studies with children living in optimally fluoridated areas are necessary to observe the effect of chewing gum which contains NaMFP on the plasma fluoride levels.

**Conclusion:**
This study indicated that:
1- The fluoride concentration in saliva samples after the use of chewing gum which contains (NaMFP ) was significantly higher than that observed after
the use of chewing gum which contains (NaF) in all the experimental times. The fluoride release during the experiment was 0.4 mg for chewing gum which contains (NaMFP) and 0.041 mg for chewing gum which contains (NaF).

2- The high fluoride concentration in saliva after the use of chewing gum which contains (NaMFP) may be important on dental caries prevention in children or adults, especially for individuals with compromised salivary flow or the ones who live in deficient fluoride areas. However, further clinical research is necessary to clarify this issue.

3- The use of chewing gum which contains (NaMFP) should be avoided by children at the age of risk for dental fluorosis because the fluoride release varied from 57% to 23.8% of the maximum recommended daily intake for children aged 1 and 7 years old, respectively

4- The use of fluoridated gum should be used to patient with high risk to caries.

References:


21- Correia, Sampaio, F.; Ramm, von, der, Fehr, F.; Arneberg, P.; Petrucci, D. and Hatloy, A. (1999). Dental fluorosis and nutritional status of


Study of the prevalence of anti Glutamic Acid Decarboxylase antibody in Iraqi children and adolescent with type 1 Diabetes mellitus

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College of Medical And Health Technology, Baghdad *, National Diabetes Center (NDC), AL-Mustansirya university**.

Abstract:

Type 1 diabetes mellitus is a disease caused by the progressive and selective destruction, by autoimmune mechanisms, of pancreatic beta cells. Recent findings support this autoimmune character, and various autoimmune
markers have been described in type1 diabetes, a number of specific and non-
specific antigens have been identified. The major autoantigen involved in the
destructive process of beta-cells leading to the development of type 1 diabetes is
 glutamic acid decarboxylase (GAD).

The aim of the present study was to assess the occurrence of anti-glutamic
acid decarboxylase (Anti-GAD) antibodies in Iraqi children and adolescents
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GAD in disease duration and BMI.

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Current classification of diabetes endorsed by both the American Diabetes
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The two major classifications of diabetes are type1 diabetes, characterized by a
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Western industrialized countries, Type I diabetes is the second most common
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Type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), results from a chronic autoimmune destruction of the insulin secreting
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host to environmental agents. Autoimmune destruction of beta cells is thought to
be completely asymptomatic until 80-90% of the cells are lost. This process may
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and GAD67 (i.e. molecular weight 65,000 and 67,000 KD). Both isoforms of GAD contain a pyridoxal phosphate binding site, a cofactor required for enzymatic activity [4].

Apart from its presence in the central and peripheral nervous systems, GAD is observed only within pancreatic islet cells, epithelial cells of the fallopian tube, and spermatozoa of the testes. In terms of function, GAD is the rate-limiting enzyme in the pathway involving the conversion of glutamic acid to GABA, a major inhibitory neurotransmitter of both the central and peripheral nervous system [5].

The function of GAD within tissues other than neurons is not clear. The presence of both GAD and GABA (γ-aminobutyric acid) within islet beta cells and the presence of GABA receptors on these cells suggests that GABA is involved in paracrine signalling in the islet. The identification of GAD as a target autoantigen of Type I diabetes dates back to a report in 1982 of a 64,000 KD that was immunoprecipitated from human islets with sera from newly diagnosed Type I diabetic children. Glutamic acid decarboxylase (GAD) catalyzes the formation of gamma-aminobutyric acid (GABA), which is a major transmitter in the central nervous system. Two forms of GAD (GAD65 and GAD67) are known to be expressed in human tissues and GAD65 is predominantly expressed in pancreatic beta-cells. Recent findings revealed that GAD functions as an autoantigen in human autoimmunity, especially in insulin-dependent diabetes mellitus (IDDM). GAD is a key antigen for the development of autoimmunity against beta-cells and the production of GADAb precedes other autoantibodies such as anti-insulin antibody (IAA) and anti-tyrosine phosphatase (ICA512/IA-2Ab) prior to the clinical onset of IDDM. At onset, GADAb is detected in 50-80% of patients [6].

In European patients with type 1 DM 95% have positive glutamic acid decarboxylase (GAD65) and/or IA2 antibodies to antigens of the islets of Langerhans; especially the finding of GAD65 antibodies seems a quite stable finding after the age of 10 to 15 years in autoimmune diabetes [7].

Materials and Methods:

The study population included 60 children and adolescents (male/female: 26/34) with clinical diagnosis of type1 diabetes mellitus, followed up in national diabetic center of al-Mustansiriya university and 20 unrelated apparently healthy, age and gender matched subjects as controls. The mean age (± SD) of patients was 9.8 (± 4.7) years and mean diabetes duration was 2.7 (± 1.5) years.

The criteria for the diagnosis of type1 diabetes mellitus were: fasting plasma glucose levels of 126 mg/dL or symptoms of hyperglycemia (polyuria, polydipsia, and unexplained weight loss) with a random plasma glucose 200 mg/dL or 2-hour plasma glucose 200 mg/dL during an oral glucose tolerance test. Body mass index was calculated as weight in Kg per height (m) squared [8].
Blood glucose and HbA1c measurements

Venous blood glucose was measured using an enzymatic method (SPINREACT, Spain). The accuracy range was 0.04mg/dL to 500mg/dL. Glycosylated hemoglobin (HbA1c) was measured by using the variant hemoglobin A1C programme developed by BIO-RAD.

Anti glutamic acid decarboxylase (GAD) antibody estimation:

Enzyme-linked immunosorbent assay (ELISA) was used to detect anti-GAD antibodies (Bio-Rad). Isoform GAD65 from human recombinant glutamic acid decarboxylase was used. The assay system uses the ability of GAD65 Abs acting divalently and forming a bridge between immobilized GAD65 and liquid-phase GAD65- Biotin. In the first step GAD65 antibody from the sample bind to GAD65 coated on the microtiter plate. In a second step GAD65-Biotin binds to this complex. The bound GAD65-Biotin correlates with the amount of GAD65 Abs in patient’s serum. Unbound GAD65-Biotin is removed by washing. The bound GAD65-Biotin could be quantified by addition of Streptavidinperoxidase and a colorogenic substrate Tetramethylbenzidin (TMB) and reading the optical density (OD) at 450 nm. For the anti- GAD antibodies, the upper limit of the normal range was set at 10 IU/mL, and any greater value was considered as positive.

Statistical analysis:

Results were expressed as mean values (±SD.). The data were analyzed using the program SPSS for Windows. All P values were two-tailed, with statistical significance indicated by a value of P < 0.05.

Results:

Data demonstrated by table-1 shows the characteristics of children and adolescents patients with type1 diabetes mellitus which revealed that the number of male and female patients was 26 and 34 patients respectively. The mean age of males was 9.5±4.5 years and mean age of females was 9.7±4.8 years, while the mean age of the total number of patients was 9.8 ± 4.7 years. The mean diabetic duration was 2.5±1.7 years in males, 2.8±1.6 years in females and 2.7 ± 1.5 years in the total number of patients. The same table also shows that the body mass index (BMI) was 18.9±3.5 kg.m-2 in males, 17.9 ±3.3 kg.m-2 in females and 17.8±3 kg.m-2 in total number of patients.

<table>
<thead>
<tr>
<th>variable</th>
<th>Age ( mean ± SD ) years</th>
<th>Diabetes duration years</th>
<th>BMI (kg.m-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (26)</td>
<td>9.5±4.5</td>
<td>2.5±1.7</td>
<td>18.9±3.5</td>
</tr>
<tr>
<td>Female (34)</td>
<td>9.7±4.8</td>
<td>2.8±1.6</td>
<td>17.9±3.3</td>
</tr>
<tr>
<td>Total (60)</td>
<td>9.8±4.7</td>
<td>2.7±1.5</td>
<td>17.8±3</td>
</tr>
</tbody>
</table>

Table-1: The characteristics of type I diabetic patients included in the study.

Table-2 shows the prevalence of anti GAD antibodies which revealed that 45(75%) of patients with type1 diabetes were anti GAD positive while only...
15(25%) were anti GAD negative. On the other hand the control group showed no prevalence of the antibody with a highly significant difference when compared with the patients group (P<0.001).

<table>
<thead>
<tr>
<th>Case</th>
<th>GAD Ab positive Number (%)</th>
<th>GAD Ab negative Number (%)</th>
<th>Statistical significance (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type1 diabetes (n=60)</td>
<td>45(75%)</td>
<td>15(25%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>0(0%)</td>
<td>20(100%)</td>
<td></td>
</tr>
</tbody>
</table>

Table-2: Prevalence of Anti-GAD antibody among patients with type1 diabetes mellitus and control group.

Table -3 demonstrates a significant difference between type1 diabetic patients with anti GAD antibody and control group regarding HbA1C, BMI, random blood sugar and fasting plasma glucose. The same table also shows a significant difference between type1 diabetic patients with no anti GAD antibodies regarding HbA1C, random blood sugar and fasting blood sugar.

<table>
<thead>
<tr>
<th>parameter</th>
<th>Patients with +ve anti GAD Ab</th>
<th>Patients with -ve anti GAD Ab</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1C (%) (mean±SD)</td>
<td>10.2±2*</td>
<td>9.2±1.9*</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>BMI( kg/m2) (mean±SD)</td>
<td>17.2±2.6*</td>
<td>20±3.6</td>
<td>21.1 ± 2.1</td>
</tr>
<tr>
<td>Random blood sugar (g/dl) (mean±SD)</td>
<td>210.5±58.9*</td>
<td>229.2±66.7*</td>
<td>129.1 ± 20.4</td>
</tr>
<tr>
<td>Fasting blood sugar (g/dl) (mean±SD)</td>
<td>178.6±52.3*</td>
<td>197±66.2*</td>
<td>89.1 ± 9.2</td>
</tr>
</tbody>
</table>

Table-3: Comparison of selected parameters between groups of type1 diabetes (anti GAD +ve AND anti GAD -ve) and control group

* P < 0.05

**Discussion:**

Anti GAD antibodies have been studied repeatedly in population samples during the last number of years. They predict insulin requirement even before the clinical onset of diabetes. They also predict insulin requirement in type 2 diabetes mellitus. Moreover, they have also been used to characterize a subset of diabetic patients called latent-onset auto-immune diabetes mellitus in adults (LADA). It is known that anti-GAD is positive in more than70 % of children with recent onset of type 1 diabetes and its level seems to decrease with the duration of the disease and decreasing number of residual beta cells. Knowing
the frequency of these autoantibodies in a population is an important step for a better understanding and diagnosis of type 1 diabetes [9].

The prevalence of anti GAD antibodies in type 1 diabetes mellitus patients included in our study was 75%, which was similar to that found in Caucasian patients, however it differs from the prevalence found in Tunisian and Japanese children which was 54% and 34% respectively. The results obtained from those studies showed that the prevalence of anti GAD antibodies in normal subjects was 2.2% which also differs from our results [10,11]. Our results also differs from the results of a study conducted in Saudi Arabia which concluded that the prevalence of anti GAD antibodies was 54% in type 1 diabetes mellitus patients [12].

Glutamic acid decarboxylase 65 autoantibodies (GAD65 Abs) are present in 70-80% of newly diagnosed patients with type 1 diabetes. GAD65 Abs also occurs in a subset of adults with type 2 diabetes. These patients can have pronounced hyperglycemia, and after therapy with oral hypoglycemic agents for several months to years they may become insulin dependent [3]. According to the literature, anti-GAD prevalence among Asian groups was relatively low compared with that of Caucasians [13,14]. Anti-GAD prevalence rates are reported to be only 5–29% in Japanese, Koreans, Thais, and Chinese residents of Hong Kong [15,16]. However, the frequency of anti-GAD in our patients is higher compared with those results. High rates for anti-GAD, similar to the Caucasians, have also been reported [17, 18]. These controversial observations might be due to the different cut-off values set among laboratories or to other environmental factors that affect the disease pathogenesis, since the dietary habits and living styles are quite diverse in the areas mentioned above even within the same ethnic group.

Recent studies have concluded that the diagnostic sensitivity of GAD65, IA-2, and insulin autoantibodies varies with age at onset and sex. GAD65 antibodies are less frequent among boys developing diabetes before the age of 10 years, but in older children, teenagers, and young adults, the diagnostic sensitivity is 80% in both males and females. GAD65 antibody titers are higher and more prevalent in patients with other associated autoimmune diseases, such as thyroiditis [19].

The differences in the prevalence rates reported in various studies are probably due to a different genetic background associated with differences in the selection of patients and/or also antibody determination. The prevalence of anti-GAD antibody was higher in females than males, however Our result of gender-related anti-GAD positivity is at variance with other studies where no gender difference of GAD antibody prevalence was seen. This discrepancy might be due to the racial difference of type 1 DM pathogenesis [20].

Although not statistically significant, another interesting observation is that there is a higher frequency (57.8% vs 42.2%) of anti-GAD in females than in males. Our observation is in accordance with three other studies and supports...
the view that organ-specific endocrine autoimmunity occurs more frequently in females regardless of racial difference \[21, 22\]. However, anti-GAD was reported to be independent of sex in Caucasian type 1 DM patients \[23\]. The finding that anti-GAD in type 1 DM is gender related, being more frequent in females than in males, further supports the theory that the autoimmune responses may be operating differently in different ethnic groups and may be gender related. Patients who did not have antibodies to GAD were more obese (higher BMI) than those who had antibodies to GAD, these findings suggest that diabetes mellitus in the population is often part of a multifaceted syndrome, commonly known as the ‘metabolic syndrome. These results were in agreement with the results obtained by a study in China which concluded that patients who had antibodies to GAD had lower BMI, a higher blood pressure, higher triglyceride levels, lower HDL-cholesterol levels, and increased albuminuria \[24\].

According to the results obtained by the present study both groups of type1 diabetes (anti GAD positive and antiGAD negative) were significantly different as far as the duration of the disease is concerned (1.3±0.8 vs. 3.5±1.8years). It is known that anti-GAD is positive in more than 70 % of children with recent onset of type 1 diabetes and its level seems to decrease with the duration of the disease and decreasing number of residual beta cells \[25\].

There was no significant difference between patients with positive anti GAD antibody and those with negative anti GAD antibody regarding age, HbA1C, random blood sugar and fasting blood sugar level. These results are in agreement with other studies \[26\].

Conclusions:
1- The prevalence of anti GAD antibodies in type1 diabetes mellitus children and adolescent included in this study was 75%.
2- There is a higher frequency of anti GAD antibodies in females than males.
3- Patients with negative antiGAD antibodies had higher BMI ratio than those with positive anti GAD antibodies.

References:


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Venous blood glucose was measured using an enzymatic method (SPINREACT, Spain). The accuracy range was 0.04mg/dL to 500mg/dL. Glycosylated hemoglobin (HbA1c) was measured by using the variant hemoglobin A1C programme developed by BIO-RAD.

Anti glutamic acid decarboxylase (GAD) antibody estimation:

Enzyme-linked immunosorbent assay (ELISA) was used to detect anti-GAD antibodies (Bio-Rad). Isoform GAD65 from human recombinant glutamic acid decarboxylase was used. The assay system uses the ability of GAD65 Abs acting divalent and forming a bridge between immobilized GAD65 and liquid-phase GAD65- Biotin. In the first step GAD65 antibody from the sample bind to GAD65 coated on the microtiter plate. In a second step GAD65-Biotin binds to this complex. The bound GAD65-Biotin correlates with the amount of GAD65 Abs in patient’s serum. Unbound GAD65-Biotin is removed by washing. The bound GAD65-Biotin could be quantified by addition of Streptavidin-peroxidase and a colorogenic substrate Tetramethylbenzidin (TMB) and reading the optical density (OD) at 450 nm. For the anti-GAD antibodies, the upper limit of the normal range was set at 10 IU/mL, and any greater value was considered as positive.

Statistical analysis:

Results were expressed as mean values (±SD.). The data were analyzed using the program SPSS for Windows. All P values were two-tailed, with statistical significance indicated by a value of P < 0.05.

Results:

Data demonstrated by table-1 shows the characteristics of children and adolescents patients with type 1 diabetes mellitus which revealed that the number of male and female patients was 26 and 34 patients respectively. The mean age of males was 9.5±4.5 years and mean age of females was 9.7±4.8 years, while the mean age of the total number of patients was 9.8 ± 4.7 years. The mean diabetic duration was 2.5±1.7 years in males, 2.8±1.6 years in females and 2.7 ± 1.5 years in the total number of patients. The same table also shows that the body mass index (BMI) was 18.9±3.5 kg.m-2 in males, 17.9 ± 3.3 kg.m-2 in females and 17.8±3 kg.m-2 in total number of patients.

<table>
<thead>
<tr>
<th>variable</th>
<th>Age (mean ± SD) years</th>
<th>Diabetes duration years</th>
<th>BMI (kg.m-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (26)</td>
<td>9.5±4.5</td>
<td>2.5±1.7</td>
<td>18.9±3.5</td>
</tr>
<tr>
<td>Female (34)</td>
<td>9.7±4.8</td>
<td>2.8±1.6</td>
<td>17.9±3.3</td>
</tr>
<tr>
<td>Total (60)</td>
<td>9.8 ± 4.7</td>
<td>2.7 ± 1.5</td>
<td>17.8±3</td>
</tr>
</tbody>
</table>

Table-1: The characteristics of type 1 diabetic patients included in the study.

Table-2 shows the prevalence of anti GAD antibodies which revealed that 45(75%) of patients with type1 diabetes were anti GAD positive while only
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15(25%) were anti GAD negative. On the other hand the control group showed no prevalence of the antibody with a highly significant difference when compared with the patients group (P<0.001).

<table>
<thead>
<tr>
<th>Case</th>
<th>GAD Ab positive Number (%)</th>
<th>GAD Ab negative Number (%)</th>
<th>Statistical significance (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type1 diabetes (n=60)</td>
<td>45(75%)</td>
<td>15(25%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>0(0%)</td>
<td>20(100%)</td>
<td></td>
</tr>
</tbody>
</table>

Table-2: Prevalence of Anti-GAD antibody among patients with type 1 diabetes mellitus and control group.

Table -3 demonstrates a significant difference between type 1 diabetic patients with anti GAD antibody and control group regarding HbA1C, BMI, random blood sugar and fasting plasma glucose. The same table also shows a significant difference between type 1 diabetic patients with no anti GAD antibodies regarding HbA1C, random blood sugar and fasting blood sugar.

Table-3: Comparison of selected parameters between groups of type 1 diabetes (anti GAD +ve AND anti GAD -ve) and control group

Table: *

*P < 0.05

Discussion:

Anti GAD antibodies have been studied repeatedly in population samples during the last number of years. They predict insulin requirement even before the clinical onset of diabetes. They also predict insulin requirement in type 2 diabetes mellitus. Moreover, they have also been used to characterize a subset of diabetic patients called latent-onset auto-immune diabetes mellitus in adults (LADA). It is known that anti-GAD is positive in more than 70% of children with recent onset of type 1 diabetes and its level seems to decrease with the duration of the disease and decreasing number of residual beta cells. Knowing
the frequency of these autoantibodies in a population is an important step for a better understanding and diagnosis of type 1 diabetes [9].

The prevalence of anti GAD antibodies in type 1 diabetes mellitus patients included in our study was 75%, which was similar to that found in Caucasian patients, however it differs from the prevalence found in Tunisian and Japanese children which was 54% and 34% respectively. The results obtained from those studies showed that the prevalence of anti GAD antibodies in normal subjects was 2.2% which also differs from our results [10,11]. Our results also differ from the results of a study conducted in Saudi Arabia which concluded that the prevalence of anti GAD antibodies was 54% in type 1 diabetes mellitus patients [12].

Glutamic acid decarboxylase 65 autoantibodies (GAD65 Abs) are present in 70-80% of newly diagnosed patients with type 1 diabetes. GAD65 Abs also occurs in a subset of adults with type 2 diabetes. These patients can have pronounced hyperglycemia, and after therapy with oral hypoglycemic agents for several months to years they may become insulin dependent [3]. According to the literature, anti-GAD prevalence among Asian groups was relatively low compared with that of Caucasians [13,14]. Anti-GAD prevalence rates are reported to be only 5–29% in Japanese, Koreans, Thais, and Chinese residents of Hong Kong [15,16]. However, the frequency of anti-GAD in our patients is higher compared with those results. High rates for anti-GAD, similar to the Caucasians, have also been reported [17,18]. These controversial observations might be due to the different cut-off values set among laboratories or to other environmental factors that affect the disease pathogenesis, since the dietary habits and living styles are quite diverse in the areas mentioned above even within the same ethnic group.

Recent studies have concluded that the diagnostic sensitivity of GAD65, IA-2, and insulin autoantibodies varies with age at onset and sex. GAD65 antibodies are less frequent among boys developing diabetes before the age of 10 years, but in older children, teenagers, and young adults, the diagnostic sensitivity is 80% in both males and females. GAD65 antibody titers are higher and more prevalent in patients with other associated autoimmune diseases, such as thyroiditis [19].

The differences in the prevalence rates reported in various studies are probably due to a different genetic background associated with differences in the selection of patients and/or also antibody determination. The prevalence of anti-GAD antibody was higher in females than males, however Our result of gender-related anti-GAD positivity is at variance with other studies where no gender difference of GAD antibody prevalence was seen. This discrepancy might be due to the racial difference of type 1 DM pathogenesis [20].

Although not statistically significant, another interesting observation is that there is a higher frequency (57.8% vs 42.2%) of anti-GAD in females than in males. Our observation is in accordance with three other studies and supports
the view that organ-specific endocrine autoimmunity occurs more frequently in females regardless of racial difference\textsuperscript{[21, 22]}. However, anti-GAD was reported to be independent of sex in Caucasian type 1 DM patients\textsuperscript{[23]}. The finding that anti-GAD in type 1 DM is gender related, being more frequent in females than in males, further supports the theory that the autoimmune responses may be operating differently in different ethnic groups and may be gender related. Patients who did not have antibodies to GAD were more obese (higher BMI) than those who had antibodies to GAD, these findings suggest that diabetes mellitus in the population is often part of a multifaceted syndrome, commonly known as the ‘metabolic syndrome’. These results were in agreement with the results obtained by a study in china which concluded that patients who had antibodies to GAD had lower BMI, a higher blood pressure, higher triglyceride levels, lower HDL-cholesterol levels, and increased albuminuria\textsuperscript{[24]}.

According to the results obtained by the present study both groups of type 1 diabetes (anti GAD positive and antiGAD negative) were significantly different as far as the duration of the disease is concerned (1.3±0.8 vs. 3.5±1.8 years). It is known that anti-GAD is positive in more than 70% of children with recent onset of type 1 diabetes and its level seems to decrease with the duration of the disease and decreasing number of residual beta cells\textsuperscript{[25]}.

There was no significant difference between patients with positive anti GAD antibody and those with negative anti GAD antibody regarding age, HbA1C, random blood sugar and fasting blood sugar level. These results are in agreement with other studies\textsuperscript{[26]}.

Conclusions:
1- The prevalence of anti GAD antibodies in type 1 diabetes mellitus children and adolescent included in this study was 75%.
2- There is a higher frequency of anti GAD antibodies in females than males.
3- Patients with negative antiGAD antibodies had higher BMI ratio than those with positive anti GAD antibodies.

References:


Kala azar one of the diseases that play role in autoimmune Thyroid diseases

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الخصائص:

إن أمراض المناعة الذاتية للدرقية عبارة عن اضطرابات تحدث في الغدة الدرقية هذه الاضطرابات

نتائج عن خطأ الجهاز المناعي في عملية الاستجابة ويدل فائته يستهدف خلايا وأنسجة الجسم نفسه.

وللغرض إثبات الدور الذي يلعبه مرض كلا، أزر في أمراض الغدة الدرقية الناتجة عن أمراض المناعة

الذاتية (المرض المناعي للثايروكولوبولين والمرض المناعي الناتج عن اضطرابات تحدث في الغدة

الدرقية نتيجة اختلال الجهاز المناعي للثايروكولوبولين). (AITPO) لهذه الدراسة، قد تم التحقق في

(50) عينة من المرضى المصابين في مرض كلا، أزر (رجال ونساء وأطفال) وكانت أعمارهم تتراوح أقل

من (1-15) سنة. أخذت العينات من مختبر الصحة المركزي. تضمنت مجموعة السيطرة (25)

شخص من الأصحاء وقامت مع مجامع الدراسة من خلال الجنس والعمر. أجري اختبار فحص طفيلي

الشخصية دونوفاني في المرضى بواسطة Dip stick rk39 (AITPO) في نفس غلاف المرضى بواسطة طريقة تطور المناعي

(IFAT) في نفس غلاف المرضى بواسطة طريقة تطور المناعي (AITPO) والمرض المناعي للثايروكولوبولين

 puedo relación بين كلا من المرضى، الذين يعانون من “المرض المناعي للثايروكولوبولين” (20 من أصل 60 مريضا) و24% يعانون

من “المرض المناعي للثايروكولوبولين، ونسبة “المرض المناعي للثايروكولوبولين” + (المرض المناعي الذاتي للثايروكولوبولين +”المرض المناعي الذاتي للثايروكولوبولين) بالمعدل (0.01 < P ≤ 0.05) بالنسبة للمرض المناعي الذاتي للثايروكولوبولين servers Comparator مع مجموعة المجمعة.

إن الهدف من الدراسة هو كشف الدور المؤثر لمرض كلا، أزر في أمراض المناعة الذاتية للغدة

الدرقية.
Abstract

Autoimmune thyroid diseases (AITD) are disorders of thyroid gland caused by an immune system defect known as autoimmunity. In autoimmunity, the immune system errs in its response and targets the body's tissues and cells. To confirm the possible role of Kalaazar disease in complications of autoimmune thyroid diseases (autoimmune thyroglobulin AITG and autoimmune thyroid peroxidase AITPO) in this study, 50 patients (male and female) were investigated afflicted with Kalaazar disease, their ages of (< 1-15) years. They were taking from Central Public Health Laboratory. The control group consisted of 25 healthy subjects comparable for age and sex of study groups. The detection of *Leishmania donovani* parasite test was done by using dipstick rK39, whereas autoimmune thyroid diseases (AITG and AITPO) tests estimated in the same patients by using immunofluorescent method (IFAT). The results indicated that patients were suffering from splenomegaly, hepatomegaly and decreased in white blood cells and hemoglobin percents. The results of IFAT tests showed that 32 patients were evaluated to have AITD with percent 64% (AITG and AITPO) of all the subjects enrolled in this study: 40% of the patients have AITG (20 out of 50 patients) and 24% have AITPO (12 out of 50 patients). And the percent of (AITG +AITPO) with accompanying was (11, 22%). The study showed there was a highly significant differences (p<0.01) among patients with AITG, AITPO comparable with other groups.

The aim of this study was to detect the effective role of Kalaazar disease in autoimmune thyroid diseases.

**Key words:** Autoimmune thyroid diseases, Kalaazar disease.

Introduction:

Autoimmune diseases are the result of an individual's immune system reacting to self constituents, whatever the specific nature of the autoimmune response, highly specific reactivity of antibodies and/or T-cells is directed against external cell- surface structures, internal cytoplasmic or nuclear constituents, or against secreted products produced by cell different organs [1].

Thyroid antibodies are a type of auto antibodies. Auto antibodies are antibodies that target specific proteins that make up the body's tissues and cells. There are several types of auto-antibodies that target the thyroid gland; these include antibodies directed against thyroglobulin (TG), thyroid peroxidase (TPO), thyroxin (T4), triiodothyronin (T3), thyrotropin (thyroid stimulating hormone or TSH) and TSH receptor [1,2,3].

Autoimmune thyroid diseases are caused by infiltration of the thyroid by lymphocytes. Interestingly the lymphocytic infiltration of the thyroid can result either in destruction of the thyroid cells, resulting in an under active thyroid (a disease called Hashimotos disease), or in stimulation of the thyroid, resulting in
an overactive thyroid (a disease called Grave's disease) \[^{[4, 5]}\]. Thyroid diseases are estimated to affect as many as 10 percent of the population, and affect women seven times more of than than men. They are frequently found in families where there are other autoimmune diseases \[^{[6]}\].

Thyroglobulin antibodies (TgAbs) are circulating immunoglobulins directed against different epitopes of the thyroglobulin molecule. Thyroid microsomal antibodies (TPOAbs) are circulating immunoglobulins directed against a component of the smooth endoplasmic reticulum of thyroid cells. Recently, microsomal antigen was found to be identical or at least to contain as main component thyroid peroxidase (TPO) \[^{[7]}\]. Detectable levels of TG Abs and/or TPO Abs are mainly associated with thyroid autoimmune disorders and with thyroid cancers but low concentrations are also found in a significant percentage of the normal population \[^{[8, 9, 10]}\].

Infectious agents have been implicated in the pathogenesis of variety of autoimmune diseases included the autoimmune thyroid diseases \[^{[11]}\]. Auto-antibodies found in sera from patients with leishmaniasis include rheumatoid factors, anti-Sm, anti-RNA, anti-SSA, and anti-SSB\[^{[12, 13]}\]. Visceral leishmaniasis (VL) may present with cytopenias along with the formation of many auto antibodies and rarely with presence of mixed cryoglobulinemia, Type II, resulting an auto immune disease \[^{[14]}\]. Another study had presented a patient with (VL) who was diagnosed as having systemic lupus erythematosis (SLE) \[^{[15, 16]}\].

Leishmaniasis (Kala azar) is a disease which the clinical diversity reflects a complex interplay between the virulence of the infecting species and the host's immune response. This form of disease exhibits a helper T-cell subtypes 1 (TH1) immune response, with interleukin 2, interferon gamma and interleukin 12 as the prominent cytokines that induce disease resolution \[^{[17]}\]. Kala azar is the most sever form of the disease which is untreated, has a mortality rate of almost 100%. It is characterized by irregular bouts of fever substantial weight loss, swelling of the spleen and liver and anemia. Although people are often bitten by sand flies infected with Leishmania protozoa, most do not develop to the disease. However, among persons who are immunosuppressed (as a result of the advanced HIV infectious immunosuppressors treatment for organ transplants, have auto logical malignancy, autoimmune disease), cases quickly evolve to full clinical presentation of sever leishmaniasis \[^{[17, 18, 19, 20]}\], and in a study in Athens University medical school had reported 2 new cases of leishmaniasis involving patients with rheumatic disease who received anti tumor necrosis factor (anti-TNF) agents and this study discussed the implications of leishmaniasis in the setting of anti-TNF therapy, which is an association with in increased risk form opportunistic infection \[^{[21]}\]. In another study related with (SLE) announced, the

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\[^{[1]}\] AJPS, 2011, Vol. 10, No. 2

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missed recognition of leishmania infection in a lupus patients was lead to death, since both the omission of a specific parasite treatment and the increase of the immunosuppressive therapy, in conviction of a lupus flare, accelerate a fatal outcome [22].

Material and methods

The study include 50 patients (male and female) which was suspected with Kalaazar disease of age (< 1-15) years and 25 healthy blood donors taken as a healthy control group. The group suspected with Kalaazar disease was subjected to the following: Determination of leishmanai donovani in serum by using dipstick rK39 Rapid Immunochromatographic Strip assay kits (InBios, U.S.A.) applied as the leaflet kit, and estimation of specific auto-antibodies IgMAG (profile) for thyroid gland by using Indirect Immunofluorescence Test (IFAT). The method named BIOCHIP Mosaic from UROIMMUN Company (Germany) applied as the leaflet kit.

1- Kalaazar detect rapid test:
A- Principle:

The Kalaazar detect for VL is rapid immunochromatographic assay. It is qualitative test based on immunoassay for the detection of antibodies to VL in human serum. The membrane is pre-coated with novel recombinant VL antigen (rK39) on the test line region and chicken anti-protein A on the control line region. During testing serum sample reacts with the dye conjugate (protein A-colloidal gold conjugate) which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically by capillary action to react with recombinant VL antigen on the membrane and generates red line. The presence of this red line indicates a positive result.

B- Procedure:
- Allow the sera and buffer to reach the room temperature prior to testing.
- Add 20 micro liters of sera to the test strip in the area beneath the arrow.
- Place the test strip into test tube, or well of 96 well tissue culture plate so that the end of the strip is facing downward as indicated by the arrows on the strip.
- Add 2-3 drops 150 micro liters of chgase buffer solution provided with this test kit.
- Read the result in 10 min.

2- Indirect Immunoflorescent Antibody Test (IFAT) for the detection of anti-thyroid gland in serum:
A- Principle:

The test kit is designed exclusively for the invitro determination of humane auto-antibodies in serum or in plasma. The determination can be performed qualitatively or quantitatively. Frozen sections of Monkey thyroid gland covering the reaction areas of a BIOCHIP Slid are incubated with diluted patient samples. If the reaction is positive, specific antibodies of classes IgA,
IgG and IgM attached to the thyroid gland antigens. In a second step, the attached antibodies are stained with fluorescein-labelled anti-human antibodies and made visible with the fluorescence microscope.

B- Procedure:
- Serum or plasma was diluted at a ratio 1:10 in PBS-Tween.
- 25 ML of diluted samples were added to reaction field of the reagent tray.
- BIOCHIP slides fitted into the corresponding recesses of the reagent tray and incubated for 30 min at room temperature.
- The BIOCHIP slides rinsed with a flush of PBS-Tween and immersed immediately in a cuvate containing PBS-Tween for at least 5min.
- 20 ML of fluorescein-labelled anti-human globulin was added to each reaction field of a clean reagent tray by using stepper pipette.
- BIOCHIP slide removed from cuvate and dried with a paper towel and immediately put into the recesses of reagent tray. Incubation for 30min at room temperature.
- BIOCHIP slide rinsed with a flush of PBS-Tween by using a cuvate with PBS-Tween for at least 5min with shaking then BIOCHIP slide was counterstained with diluted drops of Evan blue.
- 10 ML of embedding medium was added per reaction field.

C- Calculation of results:
BIOCHIP slide were examined under HOX-Magnification of a fluorescent microscope. Their dark green staining identified positively labeled cells.
Titer plane technique as follow:

**Steps explained Biochips Technique**

**Statistical Analysis:**
Comparison of paired data from the groups of subjects was done using T-test (t), while correlations between groups were analyzed using person correlation coefficient (r) formula. Statistical tables including observed frequencies with their percentage. SPSS and Microsoft Excel Programs were used for T-test and correlation coefficient calculations respectively. And the validity of AITPO test from AITG test was done by ROC Curve.

**Results and Discussion:**
All the patients with Kalaazar disease had classic clinical features include high fever, hepatomegaly and splenomegaly. Major laboratory tests showed pancytopenia (decreased in white blood cells 3000-4000 cu.mm and
haemoglobin range 6.5-9 g/dl). The results of AITDs (AITPO and AITG) were present with specific auto-antibodies with titer of 1:10 by IFAT test. The demographic study showed that there were non significant differences (P>0.05) in gender of patients afflicted with Kalaazar disease; the male in both healthy control group (18.72%) and patients group (32.64%) were represented with high frequent than female with control group (7.28%) and patients group (18.36%), as noted in (Table-1), and a non significant differences (P>0.05) was showed between the age groups /years of studied group with (1-5 years) increase number and percent in both control (14.56%) & patients (28.56%), as referred in (Table -2). The results showed that the studied groups of leishmaniasis cases consisted of positive AITPO 24% (12 out of 50 patients) and positive AITG (20.40%) at the same patients comparison with controls with a highly significant differences (P<0.01), as referred in (Table-3 and 4) respectively. And the percentage of (AITG +AITPO) with accompanying was (11, 22%) of all the cases of visceral leishmaniasis, as showed in (Table -5).

<table>
<thead>
<tr>
<th>Gender</th>
<th>Studied Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Control</td>
<td>Patients</td>
</tr>
<tr>
<td>Male</td>
<td>N 18</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>% 72.0</td>
<td>64.0</td>
</tr>
<tr>
<td>Female</td>
<td>N 7</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>% 28.0</td>
<td>36.0</td>
</tr>
<tr>
<td>Total</td>
<td>N 25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>% 100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Value</th>
<th>df</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-Square</td>
<td>0.480</td>
<td>1</td>
</tr>
</tbody>
</table>

Table-1: Sex distribution of visceral leishmaniasis Patients enrolled in the study.

<table>
<thead>
<tr>
<th>Age groups/Year</th>
<th>Studied Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Control</td>
<td>Patients</td>
</tr>
<tr>
<td>&lt;1</td>
<td>N 4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>% 16.0</td>
<td>12.0</td>
</tr>
<tr>
<td>1-5</td>
<td>N 14</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>% 56.0</td>
<td>56.0</td>
</tr>
<tr>
<td>6-10</td>
<td>N 7</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>% 28.0</td>
<td>26.0</td>
</tr>
<tr>
<td>11-15</td>
<td>N 3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>% 6.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Total</td>
<td>N 25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>% 100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Value</th>
<th>df</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-Square</td>
<td>1.725</td>
<td>3</td>
</tr>
</tbody>
</table>

Table-2: Age distribution of visceral leishmaniasis patients Enrolled in the study.
Table-3: The percentage of anti-Thyroid peroxidase antibodies in sera of visceral leishmaniasis patients and control group.

<table>
<thead>
<tr>
<th>Thyroid Peroxidase (TPO)</th>
<th>Studied Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Control</td>
<td>Patients</td>
</tr>
<tr>
<td>Positive</td>
<td>N 12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>% 24.0</td>
<td>16.0</td>
</tr>
<tr>
<td>Negative</td>
<td>N 25</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>% 100.0</td>
<td>76.0</td>
</tr>
<tr>
<td>Total</td>
<td>N 25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>% 100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Value</th>
<th>df</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-Square</td>
<td>7.143</td>
<td>1</td>
</tr>
</tbody>
</table>

Table-4: The percentage of anti-Thyroglobulin antibodies in sera of visceral leishmaniasis patients and control group.

<table>
<thead>
<tr>
<th>Thyroglobulin (Tg)</th>
<th>Studied Group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Control</td>
<td>Patients</td>
</tr>
<tr>
<td>Positive</td>
<td>N 20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>% 40.0</td>
<td>26.7</td>
</tr>
<tr>
<td>Negative</td>
<td>N 25</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>% 100.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Total</td>
<td>N 25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>% 100.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Value</th>
<th>df</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-Square</td>
<td>13.636</td>
<td>1</td>
</tr>
</tbody>
</table>

Table-5: The percentage of (AITPO+AITG) antibodies with accompanying in sera of visceral leishmaniasis patients.

<table>
<thead>
<tr>
<th>Thyroid Peroxidase (TPO)</th>
<th>Thyroglobulin (Tg)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>N 11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>% 22.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Negative</td>
<td>N 9</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>% 18.0</td>
<td>58.0</td>
</tr>
<tr>
<td>Total</td>
<td>N 20</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>% 40.0</td>
<td>60.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Value</th>
<th>df</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chi-Square</td>
<td>17.562</td>
<td>1</td>
</tr>
</tbody>
</table>

The validity of AITPO test from AITG test was:
Sensitivity= 55%
Specificity =96.67%
Accuracy= 80%
As referred in table (6) and figure (1) by ROC Curve.
Validity tests | %
---|---
Sensitivity | 55
Specificity | 96.67
Accuracy | 80

Table-6: The validity of AITPO test from AITG test by ROC Curve

Figure-1: The validity of AITPO test from AITG test by ROC Curve

Figure-2: Immunofluorescent of anti –Thyroid peroxidase Abs by IFAT (Dark green staining identified positively labeled anti-Thyroid Gland Abs) HOX-Magnification.
Infectious agents have been implicated in the pathogenesis of variety of autoimmune diseases namely, rheumatic fever, SLE, myasthenia gravis, IDDM, Sjogren, syndrome and autoimmune thyroid diseases. This study discusses the pertinent data relating to the role of infecting organisms in the development of autoimmune thyroid diseases, by which infection could trigger thyroiditis [11]. This may be related to the strategies encoding leishmania antigens [24]. And/or the involvement in this immune response of CD5+ B-1 B cells that are committed to produce multispecific autoantibodies, including IgM-RF, after a weak T-cell interaction [25]. The importance of polyclonal B-cell activation for the genesis and occurrence of auto-antibodies in VL is discussed by Bohm, et al. [26]. However it is known that RF-B lymphocytes can capture immune complex and efficiently present its processed antigenic peptides for T lymphocytes [27]. Thus, RF-B cells could play an important role in the altered immune response verified in VL, including a strong auto-antibody immune response and an exuberant synthesis of antileishmanial antibodies. This could produce larger sized immune aggregates, improving parasite opsonization through Fc receptors on macrophage surface and spreading leishmania infection [28]. While another suggestion that antibodies are induced by molecular mimicry with parasitic antigens rather than by polyclonal B cell activation [13]. Recent experimental evidence suggests that parasites can not only evade immune responses actively but also exploit the hormonal microenvironment within the host to favor their establishment growth and reproduction. The benefit for parasites of hormonal exploitation is so great that they evolved structures similar to the steroid and protein hormone receptors expressed in upper vertebrates that can bind to the hormonal metabolites synthesized by the host [29]. This study had found a
relationship between AITD (TG and TPO) and VL with a specific differences (P<0.01) especially in gender with age of (<1-5) years as show in (Table -2) and figures (2, 3). This phenomenon may be related to the accumulation of antigen presenting dendritic cell (DC) and macrophage (M) in the thyroid gland followed by thyroid autoimmune reactivity, occurs in this intrathyroidal DC accumulation coincides with enhanced growth rate and metabolism of the thyrocytes, suggesting that both phenomena are related. There is a hypothesis that DC known of their super accessory regulators of thyrocyte proliferation and hormone secretion) in other endocrine systems. The clear inhibition of thyrocyte growth by splenic DC demonstrates the regulatory role DC in endocrine systems. Proinflammatory cytokines such as IL-1B and IL-6 are important mediators in this regulation. The dual role of antigen DC represents a link between the immune and endocrine system and may be explain the understanding of the initiation of the thyroid autoimmune reaction and thyroid autoimmune phenomena seen in iodine deficiency. The accumulation and cluster formation of DC and M in the thyroid gland occurs prior to thyroid auto-antibodies formation and prior to the influx large numbers of T and B cells. The accumulation of DC and M are not only acting as APC and effector's cells in host defense ,but also as cells involved in morphogenesis (wound healing and matrix repair). Effects of IL-6 and IL-1B on thyrocytes growth and function, these cytokines inhibit differentiated function such TPO expression, thyroglobulin releasing iodine uptake, and T3 secretion. In a study mentioned that a cascade of gene-inductive events mediating inflammation elimination of the invading organism and induction of T-cell memory against reinvasion. Nrump I, a gene originally identified as Ity/Lsh Bcy for its role in controlling S. typhimurium, Leishmania donovani and Mycobacterium bovis infections in mice, regulates this cascade. The structure of the Nrump I protein might relate to its function and might mediate enhanced resistance to infection but cause susceptibility to autoimmune diseases.

In conclusion, an increased AITPO and AITG is an autoimmune finding in VL that deserves future studies to elucidate their possible involvement in VL parasitic, AITD and immunopathogenesis.

Reference:
AJPS, 2011, Vol. 10, No.2

20- http://www.who.int/emc/diseases/leish/index.html
Study of Cell Mediated Immune Response Represented By T-Lymphocytes Transformation and Proliferation in Diabetic Patients Type 2

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Biotechnology college of Pharmacy/Almustansiriyyah University

Abstract:
This study included the measurement of T- Lymphocytes transformation and proliferation for 60 diabetic patients type 2 in the range of (30-70) years of the both sexes who are entered out patients clinic of AL- Mahmodia hospital. The results compared to a control of 30 healthy individuals in the same age range.

The results showed a significant decrease percent of T-Lymphocytes transformation in diabetic patients in compared to the control group.
The selection procedure was confirmed only after performing a fasting blood sugar (FBS) for both groups.

Introduction:
Diabetes is a chronic disease that occurs either when the pancreas dose not produce enough insulin or when the body can not effectively use the insulin it produces Insulin is a hormone produced by the pancreas regulates blood sugar $^{[2,9]}$.

Hyperglycemia or raised blood sugar is a common effect of uncontrolled diabetes and over time leads to serious damage to many of body's systems especially the nerves & blood vessels. $^{[9,12]}$
Type 1 diabetes: (previously known as insulin–dependent, juvenile or childhood-onset), it characterized by deficient insulin production & requires daily administration of insulin.

Type 2 diabetes: (formerly called non–insulin-dependent or adult onset) results from the body's ineffective use of insulin. Its symptoms include excessive excretion of urine (polyuria), thirst (polydipsia), constant hunger, weight loss, these symptoms are often appears slowly, as a result the disease may be diagnosed several years after onset, once complications have already arisen [10, 12].

Type 2 diabetes is a serious & costly disease affecting 90-95% of people around the world, the chronic complications of diabetes include accelerated development of cardiovascular disease end stage renal disease, loss of visual acuity and limb amputations [1, 9, 12]. Immunological disturbances of cell mediated origin are believed to initiate from T-Lymphocyte dysfunction. Recent invitro studies implicated that in type 2 diabetes mellitus inappropriate immune responses may result from the defects in the action of insulin that is required for the function of T-Lymphocytes [7].

Mature lymphocytes recirculate via blood and lymph through lymphoid tissue in a relatively quiescent state until stimulated to proliferate during, for example bacterial or viral infection. T-Cell blastic transformation stimulated by phytohaemagglutinin (PHA) and plasma levels of immunoglobulins are markedly reduced in patients with diabetes mellitus and effect reversed by insulin administration [4, 7]. The aim of the present study is to investigate the cell mediated immune response represented by T-lymphocytes transformation in diabetic patients type 2.

Materials and Methods:

Solutions:
1- Glutamic acid solution: 25 gm glutamic acid + 100 ml distilled water
2- Antibiotic Solution: 1 gm streptomycin sulphate (SDI) + 100000 IU. Crystallized penicillin (SDI) + 100 ml distilled water.
3- Hepes Solution: Prepared by flow company to use as a buffer to keep the PH (7.4-7.5), Add ass 1: 100 ml of the complete culture media.
4- Fixative solution: 3 volumes absolute methyl alcohol + 1 volume of glacial acetic acid.
5- Hypotonic Solution: 2.85 gm Kcl + 5000 ml distilled water.
6- Sodium bicarbonate solution (0.75) %: 7.5 gm NaCo3 + 100 ml distilled water.
7- Complete RPMI- 1640 media: [(10.4 gm) RPMI- 1640 media powder + 2 gm analar sodium bicarbonate + 1L. deionized distilled water]
Sample Procedure:

1- Control group:

Blood samples from the peripheral blood were taken from a group of 30 healthy individuals aged (30-70) years. The samples collected in sterile tubes contain heparin as anticoagulant agent.

2- The cases:

Blood samples from the peripheral blood of 60 patients in the range of (30-70) years of the both sexes, all the subjects were in the category of type 2 diabetes mellitus who are entered out patients clinic of AL-Mahmodia hospital during (June- October 2009). The selection procedure was confirmed only after performing a fasting blood sugar (FBS) and then distinguished the samples into diabetic and non diabetic cases.

Lymphocyte Transformation Assay:

This test includes the measurement of lymphocyte transformation and proliferation stimulating by phytohaemagglutinin (PHA)\(^8\).

2 sterile siliconized tubes were used for each blood sample each one contained 2.5 ml of complete RPMI- 1640 media and 250 Ml of heparinized blood sample. 250 Ml of PHA added for one tube & left the other tube without PHA as a control.

The 2 tubes incubated at 37 °C for 72 hrs., after incubation they centrifuged at (2000 r/m) for 10 min.

Then amount of hypotonic solution was added with vibration and incubated at 37 °C for 50 min. then centrifuged as above,(3-8) drops of fixative agent then added to the precipitate with continuous vibration, at last 5 ml of the same fixative agent were added to the precipitate and cooled at 4 °C for 10 min., The precipitation carried for 3-4 times until had a colorless suspension, drops of the same fixative agent were added for the last precipitate with vibration then 5-7 drops of the last product of the cell suspension were fall on a clean slide 60 cm. elevation to make a smear. Smear was dried and stained by Giemsan stain for 15 min. and washed with distilled water and examined after dried by microscope at power 10* 100 using oil immersion. The stimulated cells percentage was calculate by the following formula:
**Statistical analysis:**

The statistical analysis was performed using t test to compare mean values of T.lymphocytes transformation in diabetic patients type 2 with the control group. Values of p<0.005 were considered as statistically significant [7].

**Results and Discussion:**

The results of T- Lymphocytes transformation stimulated by PHA *in vitro* test showed a significant (p< 0.005) decrease in diabetic patients when compared to the control who had normal mean of T-Lymphocyte transformation percent as showed in (Table -1)

<table>
<thead>
<tr>
<th>Case</th>
<th>number</th>
<th>Mean of T- Lymph. Trans.</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes II Patients</td>
<td>60</td>
<td>48.310</td>
<td>&lt;</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>59.223</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Table-1: Lymphocyte transformation percent in diabetic and control cases

This result agreed with [4] who reported that glutamine is both an oxidative substrate an important source for synthesis of pyridine and purine nucleotides and amino sugars in lymphocytes, glutamine is well known to be required for both lymphocytes proliferation and cytokine production, glutamine oxidative decreased in diabetic lymphocytes.

Also the study [5] was agreed with this result who noticed that a high proportion of apoptotic lymphocytes in diabetic cases may explain the impaired immune function in poorly controlled diabetic patients.

It have been reported that decreased lymphocyte transformation abnormalities may exist in membrane receptors for mitogen in these cells or may reflect intracellular defects in metabolism could well be one of the mechanisms for the impaired immune function observed in diabetic type 2 patients [4, 6].

Production of IL. 2, IL. 6 and IL. 10 is dose and time- dependently suppressed by elevation in glucose concentration, high glucose levels also inhibit proliferation of peripheral mononuclear cells [4].
Immunological disturbances in type 2 diabetic individuals have an association with cell mediated responses and inappropriate T-Lymphocyte function which is vital in this pathogenic condition has a link with insulin defect \[^{3,7}\].

Adenosine deaminase plays a crucial role in lymphocyte proliferation and differentiation and shows its highest activity in T-lymphocytes. It reported that an elevation in adenosine deaminase levels in diabetic subjects when compared to controls, the high plasma adenosine deaminase activity might be due to abnormal T-lymphocyte response or proliferation may point towards a mechanism that involves its release into circulation, its elevation could be due to altered insulin related T-lymphocyte function. This may help in predicting immunological dysfunction in diabetic individuals \[^{7}\]. The results also showed that the prevalence of diabetes type 2 in females is higher than males as showed in (Table-2) and figure (1).

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>37</td>
<td>61.7</td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>38.3</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>100</td>
</tr>
</tbody>
</table>

**Table-2: Distribution according to sex in diabetic patients type 2**

This result agreed with \[^{11}\] who reported to the high percentage of female with diabetes than male, and, \[^{12}\] which projected that 55% of diabetes deaths are in women, and \[^{1}\] who noticed that the prevalence of diagnosed type 2 diabetes is slightly higher in female than in male. The combined effect of a greater number of elderly female than male in most populations and the increasing prevalence of diabetes with age is the most likely explanation for these observations \[^{11}\].

**References:**

AJPS, 2011, Vol. 10, No.2


Assay of Paracetamol in tablet form from different Manufacturing sources in Iraqi market

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Dept. of Pharmaceutical Chemistry, College of Pharmacy, University of Baghdad, Baghdad-Iraq

Abstract:
Paracetamol (Acetaminophen) is an analgesic, antipyretic used in treatment of fever and pain in adult and children. It can be assayed using HPLC to estimate the weight of the drug in the specific dosage form.

In this study we take samples of the Paracetamol (500mg) drug from five different pharmaceutical companies in the Iraqi market to make comparison study of the drug content in these samples. The study carried out by the formation of calibration curve using standard solutions of different concentrations of the external standard paracetamol USP 30, which results in
different readings of the area under the peak. The curve will follow straight line equation. The paracetamol was extracted from its tablets & then injected into HPLC system to determine the area under the peak. From the resulted area we can calculate the concentration and the weight by the straight line equation.

The results show that all of five samples are within the maximum percentage of the differences allowed according USP from (90% - 110%).

Introduction:

Paracetamol or N-(4-Hydroxyphenyl)-acetamide is one of the most popular over-the-counter analgesic and antipyretic drugs\textsuperscript{[1]}. The first observations about the analgesic and antipyretic properties of paracetamol were made back in the late nineteenth century when alternative compounds were being sought to reduce fever in the treatment of infections.

Paracetamol is available in different dosage form: tablet, capsules, drops, elixirs, suspension and suppositories. Dosage form of paracetamol and its combinations with other drugs have been listed in various pharmacopoeias.\textsuperscript{[2,3]}

Paracetamol is a white, odorless crystalline powder with a bitter taste, 4-hydroxyacetanilide or N-acetyl-p-aminophenol and in the US Pharmacopoeia it is known as acetaminophen as shown in figure (1). It is soluble in 70 parts of water (1 in 20 boiling water), 7 parts of alcohol (95%), 13 parts of acetone, 40 parts of glycerol, 9 parts of propylene glycol, 50 parts of chloroform, or 10 parts of methyl alcohol. It is also soluble in solutions of alkali hydroxides. It is insoluble in benzene and ether. A saturated aqueous solution has a pH of about 6 and is stable (half-life over 20 years) but stability decreases in acid or alkaline conditions, the paracetamol being slowly broken down into acetic acid.

![Chemical structure of Paracetamol](image)

**Figure-1: Chemical structure of Paracetamol**

Paracetamol is 4-acetamidophenol and may be represented by the following formula ($\text{C}_8\text{H}_9\text{NO}_2$), with molecular weight (151.2), pKa (9.5).

Several papers in the literature describe the assay of Paracetamol and its combination in pharmaceuticals or biological fluids. Determination of Paracetamol using electrical method has been reported\textsuperscript{[4]},

144
Aim of the study:
The aims of this study were to investigate paracetamol from different pharmaceutical companies in Iraqi market to prove that:
1- The weight of each tablet is within the range of maximum difference allowed.
2- Assay the active constituent of different samples using HPLC-UV method & comparing the results to obtain the most potent one from the tested samples.

Materials and Methods:
Methanol of HPLC grade (Batch no. 3554) is obtained from labs can TLD, unit T26 still organ, Co-dublin, Ireland. Paracetamol B.P/USP as a standard powder is manufactured by Julphar GULf Pharma (U.A.E). The other entire chemicals were used of pharmacopoeial grade.

Apparatus:
The HPLC with liquid delivery system, KNAUER (Manager type 500), Germany is equipped with auto sampler system (chromgate 3900), KNAUER, Germany. UV-Visible spectrophotometer is obtained from (PDA detector type 2800), KNAUER, Germany. Metter balance (Metter, Toledo AB 204), Switzerland and Ultrasonic mixer FRITSCH Laboratte type 17.202 are used.

Study design:
Samples:
The raw material that used for preparation of stock solution obtained from Julphar, GULF Pharma (UEA) and tested in Iraqi National center for quality control by reference standard and the result of quality was (98%). But the samples (paracetamol 500 mg tablet) were taken from the Iraqi pharmaceutical market and (Table -1) explain the data obtained concerning the proprietary name, source, M.D, E.D, batch number and average tablet weight of each sample.
Table-1: Paracetamol 500 mg tablets in the Iraqi market.

Method:

For the preparation of the calibration or the standard curve, we used external standard of different concentrations of Paracetamol U.S.P.. The stock solution was prepared from standard Paracetamol by dissolve an accurately weighed quality (50 mg) standard powder in (100 ml) of the mobile phase to obtain a solution having a known concentration of about 0.50 mg/ml [15].

From this stock solution a different dilutions (20, 50, 100, 200, 500, and 1000 μg/ml) was made respectively to be ready for HPLC study. The chromatographic procedure is carried out by using:

1 - A stainless steel column(3.9 mm, 30 cm) column that contains packing RP-8.
2 - Mobile phase is composed from degassed mixture of water and methanol (3:1)
3 - UV detector with 243 nm wave length.
4 - 10μl of each solution was injected into HPLC system (injected volume).
5 - The flow rate is about 1.5 ml/min.

After that, calibration curve was made by injected each one of these dilutions into HPLC and the area under the peak (AUP) is measured for each
injection. By plotting the concentration of Paracetamol versus its peak area we get the calibration curve as in figure (2).

![Calibration Curve of Paracetamol](image)

**Figure-2: Calibration Curve of Paracetamol.**

We find that the calibration curve will follow the straight line equation \( Y = a + bx \), and by substitution the statistical application we get the following data:

- \( a = 0.0 \) the intercept obtained to be applied in the equation.
- \( b = 14.187 \) the slope or regression coefficient
- \( r^2 = 0.9985 \) the coefficient of determination
- \( r = 0.999149 \) the correlation coefficient

Then the straight-line equation that used in the calculation is rearranged to:

\[
Y = 14.187X
\]

The highly significant linear correlation of the area on the concentration is indicated by the high value of \( r \) and \( r^2 \), which close to the highest value of perfect correlation (1.0), this will ensure that accuracy of the work and qualification of the HPLC device.

The Maximum % difference allowed (within the range), average of AUP and concentration of the five samples are shown in (Table-2).
Table-2: The Maximum % difference, AUP & Concentration of the five samples.

The chromatogram of 0.01 mg/ml concentration of standard solution of Paracetamol is shown in (figure-3).

Procedure for sample handling:

Weigh and powder 20 tablets of each type or source of the drug and transfer an accurately weighed portion, equivalent to about 100 mg of Paracetamol, to 200 ml volumetric flask. Add about 100 ml of mobile phase, and shake by mechanical means for 10 min. Dilute with mobile phase to
volume, and mix. Transfer 5 ml of this solution to a 250 ml volumetric flask, dilute with mobile phase to volume, and mix. Pass a portion of this solution through a filter having 0.5 μm or finer porosity, discarding the first 10 ml of filtrate. The final conc. that injected is 0.01 mg/ml.

Each one of the five samples is tested using the same conditions that used in the external standard in the HPLC system to get the AUP. Then the equation of straight line is applied to calculate Paracetamol concentration & its weight.

**Results:**

From the data obtained in (Table-3) in which concentration of each AUP was determined, we can calculate the weight and recovery percent of each sample compared to the standard wt which is 500 mg, also we can calculate the Relative Standard Deviation percent (RSD%) or Sample Coefficient of variation (CV) as shown in the (table-3).

<table>
<thead>
<tr>
<th>let</th>
<th>Drug Source</th>
<th>Weight of Paracetamol of each sample</th>
<th>Recovery %</th>
<th>Standard deviation</th>
<th>RSD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Paracetamol Dijla</td>
<td>488.402 mg</td>
<td>97.680</td>
<td>4.454</td>
<td>3.053</td>
</tr>
<tr>
<td>B</td>
<td>Panadol Glaxo- smithkline</td>
<td>501.23 mg</td>
<td>100.243</td>
<td>1.828</td>
<td>0.1889</td>
</tr>
<tr>
<td>C</td>
<td>Paracetamol Furat</td>
<td>509.782 mg</td>
<td>101.956</td>
<td>0.707</td>
<td>0.046</td>
</tr>
<tr>
<td>D</td>
<td>Paracetamol SDI</td>
<td>511.16 mg</td>
<td>102.232</td>
<td>4.450</td>
<td>2.915</td>
</tr>
<tr>
<td>G</td>
<td>Paracetamol Ajanta</td>
<td>490.303 mg</td>
<td>98.06</td>
<td>0.707</td>
<td>0.048</td>
</tr>
</tbody>
</table>

Table-3: Data represent the weight of Paracetamol, recovery % and RSD% of the five samples.
Discussion and Conclusions:

From the previous study we can summarize the following:
1- All of the tested tablets were within the range of “Maximum % difference allowed”.
2- The quantitative analysis was performed using HPLC with external standard method. The recoveries were close to 100% with acceptable accuracy & precision.
3- The results indicate that paracetamol tablets is accepted within the normal percentage (90%-110%) according U.S. P.30
4- The HPLC quantitative analysis procedure is fast & accurate for Paracetamol analysis & can be used for routine work.
5- The HPLC method show good accuracy and reproducibility for determination of drug in pharmaceutical dosage forms and biological fluid.
6- From the comparison of the results obtained from the tested five samples, it was found that the panadol (Glaxosmithkline) is the most effective one and close to 100% recovery as shown in the (Table-3)

References:
Study The Antibiotic Susceptibility Patterns Of *Pseudomonas aeruginosa* Isolated from Hospital Infections

Afrah Mohammed Hassan Salman  
*Collage of Pharmacy, AL-Mustansiriya University*

Abstract:  
Out of 726 clinical specimens including wound burn, ear, pus swabs, sputum and urine, 272 isolates of *Pseudomonas aeruginosa* were detected by bacteriological and biochemical tests, 163 of these isolates were from females and 109 from males.  

181 of the isolates (66.54%) were pure isolates of *P. aeruginosa*, while 91 isolates (33.45%) of *p. aeruginosa* were found mixed with pathogenic bacteria, (76.9%) of these isolates were mixed with *Klebsiella spp.*

Antibiotic sensitivity test for *p. aeruginosa* was done by using Kirby Bauer disc diffusion method to 6 kinds of available antibiotics, it appeared that *p. aeruginosa* isolates were highly resistant to most of the antibiotics.*P. aeruginosa* isolates was found to be more resistant to aminoglycoside antibiotics:- Amikacin, Gentamycin and ciprofloxacin (84.6%, 95.95%, 79.77%) respectively, and the highly resistance was to cephalaxine showing 100%
resistance followed by Ceftriaxon (96.32), surprisingly, Pipracillin showed a relatively higher sensitivity (47.79%).

Introduction:

*Pseudomonas aeruginosa* is gram negative, motile, aerobic rods that grow readily on many types of culture media at 37-42 °C. It is oxidase positive. It does not ferment carbohydrates, but many strains oxidize glucose [1].

*Pseudomonas aeruginosa* is a major human pathogen which can produce infections of wounds and burns, urinary tract infection, respiratory tract infections and skin infections. *P. aeruginosa* is an important nosocomial pathogen in hospitals because it has many virulence factors such as pili, enzymes like protease, elastase and heat labile phospholipase and toxins like endotoxin (LPS) [2,3].

*P. aeruginosa* is widely distributed in nature and isolated as an opportunistic pathogen in recurrent infections of hospitalized patient and medical staff and is commonly present in moist environments in hospitals especially among burn and wound patient with abnormal host defenses or immunodeficiency [4,1].

*P. aeruginosa* has the ability to resist multiple kinds of antibiotic [5], such as Ciprofloxacin (75%) and Ceftriaxon (86%) [6]. The present study to see infection caused by *P. aeruginosa*, and antibiotic susceptibility pattern of *P. aeruginosa* isolated from different clinical specimens.

Material and Methods:

A total of (726) clinical specimens including (wound, burn, ear, pus) swabs, sputum and urine samples were obtained from AL-Kindy hospital from January to December-2008.

All specimens were plated on blood agar, MacConky agar, and incubated overnight at 37°C aerobically for 48 hr. [7].

Pathogenic bacteria that isolated were identified by cell morphology, colony morphology and relevant biochemical tests according to the procedure described, also EPI system was used (Oxide) [8].

Antimicrobials susceptibility test was performed to antibiotics (that available in the hospital) on Muller-Hinton agar by the standard disc diffusion method using 6 kinds of discs (Amikacin 30 Mcg, Gentamycin 10 Mcg, Ciprofloxacin 5 Mcg, Ceftriaxon 30 Mcg, Pipercillin 100 Mcg, Cephalexin 30 Mcg) by Kirby Bauer method [9,10], compared with standard strain of *P. aeruginosa* ATCC (27853) for antibiotic susceptibility test.

Results:

A total of 272 isolates of *P. aeruginosa* were isolated from (726) clinical samples of which 244 (89.7 %) were from admitted patients and 28 (10.3 %)
AJPS, 2011, Vol. 10, No.2

were from out patients, 163 isolates (59.93%) of the isolates were from females while 109 isolates (40.07%) from males. The clinical specimens including wound burn, ear, pus swabs, sputum and urine. About 91.544 percent (n= 249) of the total samples were from wound, burn swabs, and about 6.25% (n=17) were from ear swabs, 1.48% (n =4) were isolated from sputum and 0.367% (n=1) from both pus swab and urine sample. The incidences of *P. aeruginosa* among specimens are show in table-1.

<table>
<thead>
<tr>
<th>specimen</th>
<th>No. of isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound,burn swab</td>
<td>249</td>
<td>91.54</td>
</tr>
<tr>
<td>Ear swab</td>
<td>17</td>
<td>6.25</td>
</tr>
<tr>
<td>sputum</td>
<td>4</td>
<td>1.48</td>
</tr>
<tr>
<td>pus</td>
<td>1</td>
<td>0.37</td>
</tr>
<tr>
<td>urine</td>
<td>1</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table-1: Incidence of *P. aeruginosa* from 726 clinical specimens.

From 272 isolates, 181 (66.54%) were pure isolates of *P. aeruginosa* and the rest 91 (33.45%) were mixed with other pathogenic bacteria, (76.9%) of them were mixed with *Klebsiella* spp and 8 isolates (8.8%), 6 isolates (6.6%), 4 isolates (4.4%), 3 isolates (3.3%) were mixed with *Escherichia coli*, *Enterobacter* spp, *Staphylococcus aureus* and *proteus* spp respectively as in table-2.

<table>
<thead>
<tr>
<th>Name of pathogenic bacteria</th>
<th>No. of isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Klebsiella</em> spp</td>
<td>70</td>
<td>76.9</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>8</td>
<td>8.8</td>
</tr>
<tr>
<td><em>Enterobacter</em> spp.</td>
<td>6</td>
<td>6.6</td>
</tr>
<tr>
<td><em>Staphylococcus</em> <em>aureus</em></td>
<td>4</td>
<td>4.4</td>
</tr>
<tr>
<td><em>Proteus</em> <em>spp.</em></td>
<td>3</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table-2: The number and percentage of *P. aeruginos* isolates mixed with other pathogenic bacteria.

The results in (Table-3) showed that All the isolates of *P. aeruginosa* were resistant to cephalaxine (100%), and also show resistance to aminoglycosides antibiotics, (84.6%) to Amikacin, (95.95%) to Gentamycin and (79.77%) to Ciprofloxacin. The study shows a significantly high resistance to Ceftriaxon (96.32%).

The results also showed that *P. aeruginosais* was more sensitive to Piperacillin (47.79%).
Table-3: Antibiotic susceptibility pattern of 272 isolates of *Pseudomonas aeruginosa*.

### Table-3: Antibiotic susceptibility pattern of 272 isolates of *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant isolates</th>
<th>Sensitive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Amikacin</td>
<td>230</td>
<td>84.6</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>261</td>
<td>95.95</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>217</td>
<td>79.77</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>142</td>
<td>52.21</td>
</tr>
<tr>
<td>Ceftriaxon</td>
<td>262</td>
<td>96.32</td>
</tr>
<tr>
<td>Cephallexin</td>
<td>272</td>
<td>100</td>
</tr>
</tbody>
</table>

Discussion:

In the present study the percentage of *P. aeruginosa* isolates were significantly high because 91.54 % (n=249) isolates of the total samples were isolated from wound burn swabs, this goes to confirm that *P. aeruginosa* is a major factor in the etiology of burn wound infection. This finding was agreement with [11,12,13,14], because this bacterium needs a minimal nutritional requirement and it is a nosocomial pathogen that can thrive in moist environment like (water bath, shower), that enable its transfers and spread between different patient in hospital which can produces infections of wounds and burns [1], in addition to that *P. aeruginosa* has many virulence factor that enable its persistence in burn unit of hospital [15].

70 isolate (76.9%) of *P. aeruginosa* were mixed with *Klebsiella spp* and most of these isolates from burn specimens, a previous study had reported the relationship between this pathogenic bacteria (*Klebsiella*) and epidemic nosocomial infection [16], and this opportunistic pathogenic bacteria can cause many infection such as UTI, skin and wound infection [17].

The isolates showed high antimicrobial resistance to aminoglycosides and this results was agreement with [14,6], the high resistance to aminoglycosids may due to the modification in target site including a mutation that change in ribosomal unit (16 Sr RNA), modification enzyme by the production of aminoglycosids-modifying enzymes includes: Aminoglycosids Phosphotransferases(APHS), Aminoglycoside acetyl transferase (AAGS) and Aminoglycoside nucleotidyl transferases (ANTS) [18].

*P. aeruginosa* isolates were moderate sensitive (47.79%) to Piperacillin this results is disagreement with Chang *et.al* [19], Piperacillin is more effective against aerobic gram-negative rods, especially *Pseudomonas* [1].

The relatively high resistance of *P. aeruginosa* isolates to commonly used antibiotics, was because most of them still serve as first line drug, and the excessive and randomly consumption of wide broad spectrum antibiotics without proper medical prescription of. So to prevent the spreading of multidrug
resistant bacteria routine sensitivity screening of antibiotics before prescription is suggested, that will help to choose the suitable and effective drug.

References:


Evaluation of Selenium and Chloride levels in both sera and saliva samples in renal stone former patients

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*** College of Pharmacy, Al-Mustansiriya University.

Abstract:

The study included 40 patients (27 males and 13 females) with renal and ureteric stones were submitted to lithotripsy unit in Al-Yarmook Hospital, Baghdad-Iraq, their mean ages were (39±14) and 14 apparently healthy controls were enrolled in this study their mean ages were (38±12).

The study objected to evaluate the serum and saliva levels of selenium (Se) and Chloride (Cl) in patients with stones and healthy as controls to find a possible relation between these elements and stone formation.

Selenium and chloride were measured both in sera and saliva, the results showed that the mean value of serum and saliva selenium levels in stone former patients were statistically significant lower compared with healthy controls (p<0.0001, 0.002) respectively, while the mean value serum chloride level in stone former patients were significantly higher than healthy controls (p< 0.018).
This study showed that we can use serum and saliva selenium and serum chloride as a biomarker in renal stone former patients.

Keywords: Urolithiasis, Selenium, Chloride.

Introduction:
Urolithiasis is a common recurring disorder and certain intrinsic and extrinsic factors may be linked in the genesis of urinary calculi. Several studies have been done to determine the risk factors associated with urolithiasis so that preventive measures can be undertaken to prevent stone formation, however the exact etiology of urinary stones still remain unknown.

Urinary stones are similar to arteriosclerosis, the calcification that occurs in arteriosclerosis is inhibited by antioxidants. Oxalate and calcium concentration can be reduced and the process of crystallation can be inhibited by selenium which acts as nephroprotective antioxidant.

Selenium deficiency has been found to induce renal calcification, which may be primarily induced by injury of proximal tubule via oxidative stress. Supplementation of selenium and vitamin E prevents hyperoxaluria in experimental urolithic rats by decreasing the level of lipid peroxidation and the activities of oxalate synthesizing enzymes like lactate dehydrogenases, xanthine oxidase.

Selenium is trace element that is essential in small amounts. Humans and animals require selenium for the function of a number of selenium dependent enzymes, such as glutathione peroxidase, which is antioxidant enzyme that reduces potentially damaging reactive oxygen species. Recently, one of the stone formation inhibitor studied is selenium which could be stuck onto the crystal surface and would inhibit induction of new crystals, growth and aggregation.

Chloride is the major extracellular anion; it is significantly involved in maintenance of water distribution, osmotic pressure, and anion–cation balance in the extracellular fluid compartment. Chloride has a role in nephrolithiasis, can function in both Cl⁻/HCO₃⁻ exchange and Cl⁻/oxalate exchange modes. Moreover, mutations in chloride channels have been identified in the hypercalciuric nephrolithiasis disorder.

Materials and Methods:
Patient selection:
A total of 40 stone former patients (renal and ureteric) were candidate for shock wave lithotripsy and 14 healthy controls were enrolled in this study between February 2009 and January 2010. The patients and controls characteristics are summarized in table-1.
Patient assessment:

At initial presentation, blood and saliva samples for selenium and chloride were determined by atomic absorption method In Ibn Sina Labs. Patient history: all patients were free from associated morbidities apart from urinary stone, diagnosis of renal stone was depend on X-rays and Ultrasound imaging, the patients have no other diseases and take no drugs, duration of renal stone cannot be detected, all the patients have normal renal function (normal serum creatinine). Blood and saliva were collected from the patients in the lithotripsy unit in AL-Yarmouk Teaching Hospital, these samples initially were collected in plane tubes, and sera were separated and stored in deep freeze (-20°C) in the labs of the hospital.

Serum and saliva selenium were measured by using atomic absorption spectrophotometer 680AA model (Shimadzu), whereas serum and saliva chloride were measured by tittering process using silver nitrate as titrate. All these measurements were performed in Ibn Sina Labs, Ministry of Industry & minerals.

Statistical analysis:

All statistical measurements were done by using student t-test; (p < 0.05) were used as significant value.

Results:

In this study, the ages of patients and controls were nearly comparable, just one patient had bilateral renal stone were included in our study (table-1). Serum and saliva selenium concentrations in stone former patients were significantly lower as compared to control subjects (p<0.0001, 0.002 respectively), (table-2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (range)</td>
<td>15-68</td>
<td>14-58</td>
</tr>
<tr>
<td>Sex (no.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>27</td>
<td>12</td>
</tr>
<tr>
<td>female</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Renal / ureter stones</td>
<td>34/6</td>
<td></td>
</tr>
<tr>
<td>Right/left renal stones</td>
<td>25/14</td>
<td></td>
</tr>
<tr>
<td>Bilateral renal stones</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table-1: Demographic and baseline characteristics of patients & controls

Table-2: Selenium measurements in stone former patients & control subjects

The figure-1 revealed the ability of detecting the deficiency of selenium in both serum and saliva of stone former patients as compared to control subjects.
Figure-1: Selenium level (ppm) in the serum & saliva of patients compared to those of controls

We found in this study that serum chloride level in stone former patients was significantly higher than in controls (p-value 0.018), whereas the levels in saliva were not significant (p-value 0.144) as shown in table-3.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>3.752±0.975</td>
<td>4.096±0.198</td>
<td>0.018</td>
</tr>
<tr>
<td>Saliva</td>
<td>0.599±0.157</td>
<td>0.469±0.197</td>
<td>0.144</td>
</tr>
<tr>
<td>p-value</td>
<td></td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

Table-3: Chloride measurements in stone former patients & control subjects

In tables 2 and 3, revealed significant values in selenium and chloride measured in serum and saliva among the same patients (p< 0.0005, 0.0001 respectively). According to figure-2, the evaluation of chloride in serum is more reliable than in saliva of stone former patients.
Figure-2: Chloride level (ppm) in the serum & saliva of patients compared to those of controls.

Discussion:

The role of trace elements in lithogenesis is still unclear [1]. The long list of stone inhibitors includes ionic and macromolecular moieties, some being produced within the nephron in response to lithogenic insults, and some affecting not only crystallization but also crystal cell adherence. Crystal trapping is believed to anticipate a renal stone [13]. It is considered that many factors may play a role in urolithiasis. Experimental and clinical studies have shown that Selenium has an inhibitory effect on urolithiasis [7,8].

In this study the concentrations of selenium in serum and saliva in stone former were statistically significantly lower than healthy control individuals (p<0.0001, 0.002). This finding might indicate that selenium has a role in urolithiasis.

Selenium is one of stone formation inhibitor studied which could be stuck onto the crystal surface and would inhibit induction of new crystals, growth and aggregation [9].
It was found that urinary excretion of oxalate and calcium were normalized with selenium supplement \[7,8\]. Selenium administration cause a reduction in serum calcium level may be in part due to changes in calcium filtered load, changes in glomerular filtration rate or interference with calcium absorption from the gut \[14\].

Creatinine clearance increase significantly after Selenium supplementation this suggests appositive influence of selenium on glomerular filtration rate and selenium might be involved in the vascular regulatory mechanism of the kidney \[15\].

One of hypothesis of stone formation found that oxalate-induced membrane injury was mediated by lipid peroxidation reaction through the generation of oxygen free radicals. Membrane injury facilitated the fixation of calcium oxalate crystals and subsequent growth into kidney stones \[16\]. Moreover, the presence of selenium in supplement increases the antioxidant activity \[17\].

**References:**
Abstract:
Tinidazole (TZ) is an antibacterial drug used for treatment of periodontitis. More benefit may be obtained by the application of a localized oral drug delivery system consist of mucoadhesive polymers which increase the contact time between the base and the oral tissue. Different gel formulations were prepared using the bioadhesive polymers carbomer 941, sodium carboxymethylcellulose (SCMC), and guar gum. The influence of polymer type and polymers blend in varying ratio on the viscosity, bioadhesive strength, swelling index and drug release were evaluated. SCMC based gel showed fastest release in comparison with carbomer and guar gum based gel. Using polymer blend of SCMC with either carbomer or guar gum resulted in a modification of both release and physical properties. The release of TZ was decreased with increasing amount of carbomer and guar gum and decreasing amount of SCMC. And increasing the viscosity of the gel formulations resulted in a retardation effect on the release of the drug. The study also showed that...
formulas containing carbomer exhibited maximum swelling values with lower release rates and best mucoadhesion.

**Keyword:** tinidazole, mucoadhesive oral gel, carbomer, sodium carboxymethylcellulose, guar gum.

**Introduction:**

Bacterial plaque is believed to be the main etiological agent of periodontal disease. The sub gingival micro flora associated with destructive periodontitis is predominantly Gram-negative and anaerobic [1]. Large doses of systemic antibiotics must be taken in order to achieve sufficient concentration in the gingival crevicular fluid of the periodontal pocket; this may raises a number of issues, like bacterial resistance to administered antibiotics and unpleasant or toxic side effects [2]. The lack of drug retention in the periodontal pocket is probably the chief reason for these mixed results. The attractiveness of treating periodontal disease using the sustained release of antimicrobial agent is based on maintaining effectively high level in the gingival crevicular fluid [3].

Local delivery in the oral cavity had particular application in the treatment of tooth ache, periodontal diseases and bacterial infection [4]. Of all topical formulations available, gel bases which is likely to stay in the mucosal surface seems to be the most suitable vehicle for drug delivery to the oral cavity tissue.

To increase the adherence between the bases and the oral tissues, polymers with bioadhesive properties are selected as gelling agent [5, 6]. Mucoadhesive polymers of natural, semisynthetic or synthetic origin are able to form hydrogel which swell in presence of water and physically entrap drug molecule for subsequent slow release by diffusion or erosion. Among bioadhesive polymer, poly (acrylic acid)-based polymers like carbopol and polycarboxphil, cellulose derivatives like sodium carboxymethylcellulose, hydroxy propylmethyl cellulose and methyl cellulose and natural gum like xanthan gum and guar gum [4]. Several buccal devices were formulated with tinidazole which is close analogue to metronidazole for treatment of periodontitis like tinidazole dental implant and tinidazole stilus [7,8].

In the present work mucoadhesive gels of tinidazole that adhere with gums for a prolonged period of time were prepared. The mucoadhesive gels were prepared by using hydrophilic polymers (carbopol-941, guar gum and sodium carboxymethylcellulose). The effect of type and polymer ratio on the mucoadhesiveness and release of tinidazole were studied in addition to evaluating the swelling index and the rheological behavior of the prepared gels.

**Material and Method:**

Tinidazole (TZ) (Sigma Chemical Co), Carbomer 941 (Goodrich, USA), Sodium carboxymethylcellulose (SCMC) (BDHchemical, Ltd, Pool, England), Guar gum, Methylparaben (MP) and Propylp-araben (PP) (Samra Drug
Industries), Triethanolamine (TEA) (Hopkins and Williams, England), Mannitol (E. Merck, Darmstad).

**Preparation of Gel:**

1- **Preparation of single polymer gel:**

0.2% w/w MP, 0.02% w/w PP and 2% w/w mannitol were dissolved in distilled water, 4% w/w of each of carbomer, guar gum and SCMC powder were added slowly to the previous solution under continuous stirring at 50 rpm. A (0.5) ml of TEA was added to carbomer dispersion with continuous stirring till transparent clear gel was formed. The resultant gel masses were left over night at room temperature for complete swelling. Part of the prepared gel was added to 1% of the powdered drug with gentle stirring to produce a smooth layer of the gel. The rest of the gel was added gradually portion by portion with continuous gentle stirring to avoid air entrapment till a homogenous dispersion was obtained.[9,10].

2- **Preparation of combination polymer gel:**

Different gel formulations were prepared with various ratios of carbomer to SCMC and guar gum to SCMC of 3:1, 1:1 and 1:3 by the same method mentioned previously. Different formulations of TZ mucoadhesive oral gel are given in (table-1).

**pH determination:**

Accurately 2.5 gram of gel was weighed and dispersed in 25 ml of water and then the pH was measured[11].

**Physical examination:**

The prepared gel formulations were inspected visually for color, homogeneity and consistency.

**Drug content analysis:**

A modified assay method was adopted to determine the drug content of the prepared gel.

1 gram gel was accurately weighed and dissolved in 25 ml methanol in tightly closed volumetric flask. The closed flask was shaken for 10 minute, then the mixture was filtered. The volume of filtrate was made up to 50 ml with methanol. One ml of the above solution was further diluted to 25 ml with methanol. The total TZ content was determine by comparing the U.V. absorbance of the resultant solution at a wave length of 310 nm to the standard curve of TZ in methanol[12].

**Rheological study:**

Gel viscosity measurement was evaluated at 25°C using rotation viscometer. The samples were sheared with spindle R7 by applying increasing value of shear rate over the range of speed setting from 1.5 to 12 rpm. The sample was allowed to settle for 5 minutes prior to taken the reading, then in a descending order[13].
Determination of the mucoadhesive force:

The mucoadhesive potential of each formulation was determined by measuring the force required to detach the formulation from buccal mucosal tissue using modified physical balance method\[14\]. A section of buccal mucosa was cut from the sheep buccal cavity and instantly fixed with the mucosal side out, on to glass vial using a rubber band. The diameter of each exposed mucosal membrane was (1.8) cm. The vial with buccal tissue were stored at 37°C for 10 minutes. Another vial with mucosal tissue was connected to the left side of two-arm balance and the stored vial was fixed on a height adjustable-pan. To the exposed tissue on this vial, a constant amount of 0.1 gram gel was applied. The height of the vial was adjusted so that the gel could adhere to the mucosal tissue of both vial. A force of 0.1 N was applied for 2 minutes to ensure intimate contact between the tissue and the sample. After removal of preload force, water was added slowly to previously weighed beaker placed on the right hand pan until vial get detach. The bioadhesive force expressed as the detachment stress in dyne/cm², was determined from the minimal weight that detach the tissue from the surface of each formulation using the following equation \[15,16\].

\[
\text{Detachment stress (dyne/cm}^2\text{)} = \frac{m.g}{A} \quad \text{......eq (1)}
\]

Where:
m: the weight added to the balance in gram
g: acceleration due to gravity taken as 980 cm/sec²
A: area of tissue exposed

Swelling index study:

In this study, 1 gram sample was put into a stainless steel basket with 200 mesh of aperture, and weighed. The basket was then placed in 100 ml distilled water, allowing the gel to swell at 25°C for 6 hours. The basket was periodically weighed after removing the excess water on the surface with filter paper:

\[
\text{Swelling} \% = \frac{[W_t - W_0] \times 100}{W_0} \quad \text{......eq (2)}
\]

Where \(W_t\) is the weight of basket at time \(t\) and \(W_0\) is the initial weight of the basket \[17\].

In vitro study of drug release:

The release study was carried out with USP dissolution apparatus type I at 50 rpm and 100 ml phosphate buffer pH6.8 maintained at 37°C. The apparatus was slightly modified to overcome the small volume of the dissolution medium using a suitable glass beaker inside the dissolution flask. A basket of 2.5 cm in diameter was enclosed with multifold filter paper filled with 1 gm of TZ gel, immersed to about 1 cm of its surface in the dissolution medium \[18\]. Samples collected (1ml) at 15, 30, 45 minutes 1, 2, 3, 4, 5 and 6 hours and replaced immediately with the same volume of dissolution medium. The samples following suitabl dilution were assayed spectrophotometrically at 320nm \[12\].
Stability study:  
The selected formula was stored in well-sealed glass vials for a period of 4 months at 40°C, 50°C and at 4°C. at predetermined intervals, samples were collected and drug content was analysed to predict the expiration date. The physical properties were also evaluated.

Results and Discussion:  
Physicochemical properties:  
The physicochemical properties of the prepared formulation are shown in (table-1). It is clearly evident that all the gel formulations are homogenous, smooth with acceptable consistency. The physical appearance of the prepared gel was transparent or opaque in nature with pH range of 6.4-6.93 which lies in pH range of the oral mucosa which is reported to be between 6.2-7.4. Furthermore, the three buffer systems of the salivary system are able to maintain a non-harmful pH (6.0-7.5) in the oral cavity\[19]\]. Thus all the formulation considered to be not acidic, so it may not cause any damage to the hard and soft oral tissue.

Rheological study:  
All the gel formulation demonstrated pseudoplastic flow with thixotropy. The flow curve of formula F6 is shown in (figure-1) as an example of the flow behavior of the gel formulations. Shear thinning phenomenon, an advantageous property of buccal gel, was observed for all the gel tested. In this flow the molecule at rest entangled with the association of the immobilized solvent. Under the influence of shear, the molecule tends to become disentangled and align themselves in the direction of flow. The molecules thus offer less resistance to flow and this together with the release of entrapped water account for the lower viscosity\[20]\.

The viscosity of different gel bases is described in (table-2). The apparent viscosity values were used as a measure of gel consistency. Although solid content were equal, these values appear to be markedly different, revealing variability in net work structure\[21]\]. Carbomer based gel showed higher viscosity values indicating higher consistency which may be due to its cross-linked structure and the molecular weight between cross link, reflects this rheological behavior\[22]\.

Mucoadhesive force:  
The ex-vivo mucoadhesive property of the gels were determined using sheep buccal mucosa. Mucoadhesive force in term of detachment stress, (table-2), indicated that the bioadhesive force for carbomer is much more than SCMC and guar gum which may be attributed to the high viscosity of carbomer based gel\[23]\. Carbomer also has a very high percentage (58-68) of carboxylic group in its chemical structure that gradually undergo hydrogen bonding with sugar residue in the oligosaccharide chain in the mucous membrane resulting in the formation of strengthened network between polymer and mucus. In addition
may also adopt more favorable macromolecule confirmation with the accessibility of its functional group for hydrogen bonding, while other polymers only undergo superficial bioadhesion\(^{[24,25]}\). On the other hand, the charge of the polymer tended to affect the mucoadhesive force, where nonionic polymer appear to undergo a smaller degree of adhesion compared to anionic polymer\(^{[26]}\). This explain why the formula (F1), anionic polymer based gel, had mucoadhesive force higher than formula (F2), nonionic polymer based gel\(^{[27]}\).

**Swelling index:**

The swelling index as a function of time is shown in (table-3). As the time increase, the swelling index increase, because weight gain by the gel increased proportionally with rate of hydration, later on the swelling index of the formulas (F2, F3, F7, F8 and F9) decrease gradually due to dissolution of outer most layer of the gel in the dissolution medium\(^{[28]}\). The direct relation ship was observed between swelling index and carbomer concentration, this could be attributed to the ionization of the carboxylated moiety at the pH environment of the dissolution medium. Ionization of carbomer leads to the development of negative charges along the backbone of the polymer. Repulsion of the like charges uncoils the polymer into an extended structure. The counter ion diffusion inside the gel creates an additional osmotic pressure differences across the gel leading to a considerable swelling of the polymer\(^{[29]}\). The swelling of formula F3 is relatively higher than the formula F2 since water causes ionization of carboxylic group of SCMC with subsequent relaxation and repulsion of the polymer chain that result in an increase in water penetration and hence increase in swelling index by time, while guar gum is neutral polymer\(^{[27,30]}\). On the other hand the swelling index increases in the same order of increasing viscosity. These findings are in agreement with those obtained by Pakah et al., who reported that the water absorption rate increases as the viscosity of the polymer increases \(^{[31]}\).

**The in vitro release of TZ:**

**Effect of polymer type:**

Gels with particular polymer were prepared to study the effect of polymer type on the release profile. (Figure-2) show the release profile of TZ from formulas (F1, F2 and F3). Being an anionic and water soluble, SCMC based gel (formula F3) released more than 94% of TZ within 3 hours, and approximately 30-40% of drug released within 30 minutes. This formula showed burst release due to rapid dissolution of the gelling polymer in the pH of the dissolution medium. Carbomer and guar gum gel showed integrity beyond 6 hours and did not dissolve completely even after 6 hours. More retardant effect was obtained with carbomer this may be attributed to the highest viscosity and swelling of this gel than other tested gel preparations. It was demonstrated that under the condition of the dissolution medium swelling of carbomer increases rapidly and consequently the viscosity. In contrary, guar gum gel showed relatively higher
percentage of drug release than carbomer gel, since it is non ionic polymer and so the pH has no effect on their swelling and viscosity\[^9, 27\].

**Effect of polymer combination:**

To obtain adequate release of the drug, it is thought to prepare formulas containing mixture of SCMC (fast drug release polymer) and guar gum or carbomer (slow drug release polymers). (Figures-3 and 4) show the release profile TZ from gel with combination polymers. F8and F9 showed burst release (approximately 30-40% of drug release within 30 minutes) and almost complete drug release within 5 hours. These gel preparations where unable to give prolonged action and maintain the therapeutic action for longer period of time. The drug release rate constants (table-4) appear to decrease significantly (p<0.05) with decreasing amount of SCMC and increasing amount of guar gum. The inclusion of higher percentage of guar gum (F7) provide prolonged release of drug through its property of slow eroding and as a rigid gel structure forming agent\[^10\]. The release rate constants for carbomer: SCMC gels decreased significantly (p<0.05) with decreasing amount of SCMC and increasing amount of carbomer, this could be described as the corresponding reduction in the number and dimension of the channel by increasing viscosities of the formulations\[^3\]. As it is illustrated in (table-3) and (figure-3) although gels containing carbomer:SCMC exhibit maximum swelling, they showed lower rate of release which could be attributed to higher hydrophillicity and water uptake of carbomer which produce water swollen gel that may substantially reduce the penetration of the dissolution medium into the gel and as the result the drug release \[^33\].

**Kinetics of drug release:**

The in-vitro release of TZ generated linear relationship between the amount released and square root of time as shown in (figure-5) with good correlation of coefficient(r^2) over 0.99 for all formulations (table-4), indicating that the release kinetic followed Higuchi-diffusion model \[^34\].

\[
F = k \sqrt{t} \quad \cdots \text{eq}(3)
\]

Where F is the fraction of drug released, k is the release constant, and t is the time.

Diffusion is related to transport of the drug from the gel matrix into the surrounding in vitro dissolution medium and it depend on drug concentration. As gradient varies, the drug is released and the distance for diffusion increase. This could explain why the drug is diffuses at a comparatively slower rate as the distance for diffusion increases\[^35\]. Based on the release rate constants, formulas F6 and F7 showed long term controlled release kinetics, while formulas F4 and F5 showed slow release kinetics.

**Stability study (effect of storage time):**

The selected formula F6 showed good physical stability, as there was no discoloration, precipitation, or any physical changes after storage. (Figure-6) shows the effect of different temperatures on the percentage of TZ remaining.
The results obtained showed linear profiles, from which the degradation rate constants \( k \) were calculated from the slopes. They were found to be \( 7.43 \times 10^3 \), \( 9.34 \times 10^3 \) and \( 3.37 \times 10^3 \) \( \text{month}^{-1} \) at 40°C, 50°C and 4°C respectively. The rate constant \( k \) at room temperature was determined by Arrhenius plot. The expiration date of formula F6 was found 1.83 years with a pH value of 6.64.

**Conclusion:**

A mucoadhesive system for the controlled release of TZ was developed by using carbomer and SCMC in appropriate ratio. The release rate of TZ from the prepared gel as well as the physical properties is affected by the type and the change in polymer mixing ratio. Lower release rate was observed by lowering the content of SCMC in carbomer: SCMC and guar gum: SCMC containing formulation. The mucoadhesive TZ oral gel containing 1% carbomer and 3% SCMC showed suitable release kinetics and adhesion property may be considered useful formula for delivery of TZ into the periodontal pocket.

<table>
<thead>
<tr>
<th>Batch codes</th>
<th>TZ</th>
<th>carborner</th>
<th>Guar gum</th>
<th>SCMC</th>
<th>MP</th>
<th>PP</th>
<th>mannitol</th>
<th>Water</th>
<th>pH</th>
<th>Physical appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1</td>
<td>4</td>
<td></td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.51</td>
<td></td>
<td>Transparent</td>
</tr>
<tr>
<td>F2</td>
<td>1</td>
<td>4</td>
<td></td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.89</td>
<td></td>
<td>opaque</td>
</tr>
<tr>
<td>F3</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.47</td>
<td></td>
<td>transparent</td>
</tr>
<tr>
<td>F4</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.44</td>
<td></td>
<td>transparent</td>
</tr>
<tr>
<td>F5</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.65</td>
<td></td>
<td>transparent</td>
</tr>
<tr>
<td>F6</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.81</td>
<td></td>
<td>transparent</td>
</tr>
<tr>
<td>F7</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.71</td>
<td></td>
<td>opaque</td>
</tr>
<tr>
<td>F8</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.89</td>
<td></td>
<td>opaque</td>
</tr>
<tr>
<td>F9</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>0.2</td>
<td>0.02</td>
<td>2</td>
<td>100</td>
<td>6.93</td>
<td></td>
<td>opaque</td>
</tr>
</tbody>
</table>

**Table-1: Formulations of TZ mucoadhesive oral gel (% W/W)**

<table>
<thead>
<tr>
<th>TZ Gel</th>
<th>( \eta_{\text{max}} ) (poises)</th>
<th>( \eta_{\text{min}} ) (poises)</th>
<th>Mucoadhesive force (dyne/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>3076.20</td>
<td>13350.00</td>
<td>9425.6</td>
</tr>
<tr>
<td>F2</td>
<td>535.55</td>
<td>1957.40</td>
<td>6125.0</td>
</tr>
<tr>
<td>F3</td>
<td>646.10</td>
<td>2103.18</td>
<td>6329.8</td>
</tr>
<tr>
<td>F4</td>
<td>4674.54</td>
<td>10250.78</td>
<td>7690.2</td>
</tr>
<tr>
<td>F5</td>
<td>2545.04</td>
<td>4727.06</td>
<td>6805.5</td>
</tr>
<tr>
<td>F6</td>
<td>1079.60</td>
<td>4311.71</td>
<td>6465.8</td>
</tr>
<tr>
<td>F7</td>
<td>270.16</td>
<td>1994.56</td>
<td>6111.0</td>
</tr>
<tr>
<td>F8</td>
<td>396.43</td>
<td>2048.12</td>
<td>5988.4</td>
</tr>
<tr>
<td>F9</td>
<td>6599.00</td>
<td>2083.39</td>
<td>5921.8</td>
</tr>
</tbody>
</table>

**Table-2: Physical evaluation of different TZ mucoadhesive oral gels**

<table>
<thead>
<tr>
<th>( \eta_{\text{max}} ) (poises)</th>
<th>( \eta_{\text{min}} ) (poises)</th>
<th>Mucoadhesive force (dyne/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>3076.20</td>
<td>9425.6</td>
</tr>
<tr>
<td>F2</td>
<td>535.55</td>
<td>6125.0</td>
</tr>
<tr>
<td>F3</td>
<td>646.10</td>
<td>6329.8</td>
</tr>
<tr>
<td>F4</td>
<td>4674.54</td>
<td>7690.2</td>
</tr>
<tr>
<td>F5</td>
<td>2545.04</td>
<td>6805.5</td>
</tr>
<tr>
<td>F6</td>
<td>1079.60</td>
<td>6465.8</td>
</tr>
<tr>
<td>F7</td>
<td>270.16</td>
<td>6111.0</td>
</tr>
<tr>
<td>F8</td>
<td>396.43</td>
<td>5988.4</td>
</tr>
<tr>
<td>F9</td>
<td>6599.00</td>
<td>5921.8</td>
</tr>
</tbody>
</table>

\( \eta_{\text{max}} \) Viscosity at high rate of shear (14.68 sec\(^{-1}\))

\( \eta_{\text{min}} \) Viscosity at low rate of shear (2.24 sec\(^{-1}\))
### Table-3: In-vitro swelling study of mucoadhesive oral gels of TZ

<table>
<thead>
<tr>
<th>Formulations code</th>
<th>K (µg. hour$^{1/2}$ ml$^{-1}$)</th>
<th>Correlation coefficient ($r^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>4.107</td>
<td>0.998</td>
</tr>
<tr>
<td>F2</td>
<td>5.734</td>
<td>0.9960</td>
</tr>
<tr>
<td>F3</td>
<td>10.463</td>
<td>0.993</td>
</tr>
<tr>
<td>F4</td>
<td>4.910</td>
<td>0.996</td>
</tr>
<tr>
<td>F5</td>
<td>5.900</td>
<td>0.996</td>
</tr>
<tr>
<td>F6</td>
<td>7.954</td>
<td>0.995</td>
</tr>
<tr>
<td>F7</td>
<td>8.496</td>
<td>0.996</td>
</tr>
<tr>
<td>F8</td>
<td>9.251</td>
<td>0.996</td>
</tr>
<tr>
<td>F9</td>
<td>9.473</td>
<td>0.984</td>
</tr>
</tbody>
</table>

**Table-4: Release rate constants for TZ mucoadhesive oral gels**

* Significant at $P<0.05$
* * Highly Significant at $P<0.001$

![Figure-1: Rheogram of formula F6 (1% carbomer:3%SCMC)](image-url)
Figure-2: The effect of polymer type on the release of TZ from mucoadhesive oral gel at pH6.8 and 37°C.

Figure-3: The effect of polymer combination on the release of TZ from (carbomer: SCMC) mucoadhesive oral gel at pH6.8 and 37°C.

Figure-4: The effect of polymer combination on the release of TZ from (guar gum: SCMC) mucoadhesive oral gel at pH6.8 and 37°C.
Figure-5: In vitro release of TZ from oral gel formulation containing different mucoadhesive gelling agent.

Figure-6: Degradation of tinidazole in formula F6 at 50°C, 40°C and 4°C

Figure-7: Arrhenius plot for expiration date estimation of tinidazole mucoadhesive oral gel (formula 6)
References:


The Effect of Aqueous Extract Cinnamon Zeylanicum Bark on the Structure and Function of the Ovary in Female Rats

Enas Waleed Shakir Al-Najjar
Dept. of Biological Science, College of Science, AL-Mustansiriyah University

Abstract:

The effect of aqueous extract cinnamon zeylanicum bark on the structure and function of ovary was studied for the females of rats physiologically and histologically.

Number of (20) white rats were used for this study, divided into two groups that treated as follows:

The first group is the control group that have got the regular water and food, while the second group has been treated with the aqueous extract cinnamon bark of concentration 5 mg/ml for a period of 15 days.

The histological study for the aqueous cinnamon bark extract treated group showed that the existence of the Primary follicles, Preantral follicles and in addition to a number of Corpora lutea that indicates the occurring of ovulation
comparing with the control group that showed only the Primordial follicles which reflects the effect of the cinnamon on the ovary activity.

The hormonal study shows also a significant increment (P<0.01) in the levels of Progesterone, Estradiol, LH, and FSH in the treated groups comparing with the control group.

The results of this study indicates the ability of the cinnamon plant to stimulate secretion of the gonadotropin and hence the activity of the ovary.

**Introduction:**

The ovary is an ovoid structure that can be divided into the outer cortex and the inner medulla [1]. Medulla consists of connective tissue and a large number of blood vessels, lymphangitis and nerves while the cortex consists of ovarian follicles and corpora lutea in various stages of development these structures are embedded in a loose connective tissue stroma [2]. A follicle is a structure containing an oocyte surrounded by specialized epithelial cells. The follicular growth and maturation is dependent on FSH from the adenohypophysis and LH is important for estrogen synthesis and ovulation [3].

Primordial follicles are found in the outer portion of the cortex, and it’s appear in stage of pre-puberty and the stage of sexual maturation at day (50th) in the ovary are characterized by the presence of primary follicles and growing follicles [4], the ovary considered as a gland of internal secretion (endocrine function) for its production of sex steroids hormones Estrogen and Progesterone [5]. The secretion of these two hormones by the ovaries is in response to hormones of anterior pituitary which are Follicle Stimulation Hormone (FSH) and Luteinizing Hormone (LH) and both are secreted in response to the under hypothalamus hormone (GnRH) , all of these hormones represent the female hormone system [5,6].

Cinnamon is one of the oldest herbal medicines known, having been mentioned in Chinese texts as long as 4,000 years ago, and is considered one of good medicinal plants where it is used in cases of colds, flu, abdominal cramps and against bacteria and fungi [7], many of the studies confirmed the using of kinds of cinnamon in the treatment of illness cases that affect the female reproductive system where the Cinnamomum zeylanicum was used for the treatment of Uterine Hemorrhage that occurs after birth and cause anemia which may led to the death of many of the mothers. It is also used in the treatment of fertility and infertility in women and men, inflammation and fibrosis of the uterus, cysts the ovary, the delay and pain menstrual cycle [9], where revealed the Japanese researches that the cinnamon stimulus for menstruation since it calls the uterus and urges bleeding menstrual and taken in India after childbirth as a contraceptive [10], the bark of cinnamon used to treat menstrual disorders and organization [11]. The medicinal effects of cinnamon oil are very powerful, and there are many uses for it [13]. A study showed that the medical influence of cinnamon because of the turbines in its essential oil, and the most important
compound of the oil is a complex compound known as the cinnamaldehyde which is reason of many pharmacologic effects. The case of irregular hormones is a common in these days and have a lot of effects, especially on the ovulation and the menstrual cycle so that the use of many herbs trying to regulators of these hormones in the body, particularly Estrogen and Progesterone, which regulate the function of the ovaries and pituitary gland, from here came the idea of this study due to the importance of medical cinnamomum and its impact on stimulating the body's physiological activity.

Materials and Methods:

Experiment was conducted on 20 white rat Rattus norvegicus (females) aged two months and weights ranging between (100-150) gm were taken from the animal house at the college of Medicine in Baghdad University and then animals were divided into two equal groups, as follows:

A: Control Group: Animals of this group continued to eat regular drinking water freely throughout the experiment.

B: Treatment Group: Animals of this group has been dosing daily for 15 days by the aqueous extract cinnamon bark with concentration of (5mg/ml) through the prepared tube oral.

- (Blood collection) the blood samples have been taken in the sixteenth day from each rat by puncture heart.
- (Hormonal assay) the hormonal study was made on the blood serum by using the method Radioimmunoassay.
- (Anesthesia) the animals were anesthetized and their ovaries removed after absolved from the surrounding tissue and tissues samples were fixed to preserve the structure of the tissue, the fixatives used was formaldehyde (10%formalin).
- (Staining by Hematoxyelin and Eosin stain) the samples passed to different concentrations of alcohols and then painted stained Hematoxyelin and Eosin to color the nuclei dark blue (Hematoxyelin) and the remaining cell components pink (Eosin) for the purpose of the Histological study and photography.
- Statistical analysis was used by using analysis of variance (One analysis of variance) when there are differences of moral and high moral conduct comparisons using the test T (T-test).

Results and Discussion:

Hormonal Assay:

The levels of the four hormones (Luteinizing hormone (LH), follicle stimulating hormone (FSH), Estradiol, Progesterone) has increased significantly (p <0.01) in the treated group of an aqueous extract cinnamon bark with a
concentration (5 mg/ml) compared with a control as in (Table -1) and measured in a radioimmunoassay (Radio Immune Assay) (RIA).

<table>
<thead>
<tr>
<th>The measured hormones in the serum.</th>
<th>Control Group (mean± SD)</th>
<th>Treated Group (mean± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH (Int. Unit/ml)</td>
<td>0.26±15.00</td>
<td>0.31±17.43**</td>
</tr>
<tr>
<td>LH (Int. Unit /ml)</td>
<td>0.15± 3.17</td>
<td>0.09±3.83**</td>
</tr>
<tr>
<td>Estradiol (pg/ml)</td>
<td>0.43 ± 10.59</td>
<td>0.63 ± 18.75**</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>0.26 ± 10.30</td>
<td>0.54±18.99**</td>
</tr>
</tbody>
</table>

Table-1: The influence of aqueous extract cinnamon bark with concentration (5 mg/ml) in the hormones FSH, LH, Estradiol, Progesterone in the serum of control animals and treatment.

** Significance difference <0.01

Histological Study:

The study of tissue sections stained with Hematoxylin - Eosin (HE) shows clear histological changes as follows:

1- For the control group a presence inactive Primordial follicle within the cortex as shown figure-1.

2- Treated group with aqueous extract cinnamon bark with concentration (5 mg/ml), the growth and development of prenatal follicles and Antral follicles was clearly appeared in addition to the appearance of vesicle Mature Graafian and forming corpus luteum as shown in figure-2 comparing with a control group.

Figure-1: A section of ovarian tissue in female non-treated control rat shows the cortex (C), containing Primordial follicles (P) and the pulp Medulla (M) (200x) (H & E)
Figure-2: Section of tissue of the female rat treated of aqueous extract cinnamon bark oncentration (5 mg / ml) for a period of 15 days, the appearance of Preantral follicles (PF), Antral follicles (AF) and vesicle Mature Graafian follicle (MG) and Corpus luteum (CL) (200x) (H & E)

The results showed that the aqueous extract cinnamon bark led to an increase in the activity of ovarian, as the high level of hormones LH, FSH due to the presence of compound within the cinnamon led to increase and improve the secretion of these two hormones \[^{18}\] since its stimulate pituitary gland to increase secretion of the follicle stimulating hormone and therefore the events of growth follicular (16), where the hormone FSH stimulates the growth of follicles, as well as increasing the level of Estradiol \[^{6}\], and this agrees with the study showed that cinnamon working to increase the level of both FSH and Estradiol hormones among women and hence the occurrence of ovulation \[^{24}\], it has been observed an increasing in the level of the Estradiol hormone in this study, which represent the occurrence of ovulation. As the studies have been shown that compounds within the cinnamon affect in the growth of follicular and improve the secretion of LH from the pituitary gland before ovulation \[^{17}\], where this hormone is also working on the maturation of the egg and the occurrence of ovulation too \[^{4}\], since Wechler the essential oil in the cinnamon cause the occurrence of ovulation \[^{25}\].

This is matches with the histological study, where the appearance of follicles reflecting the cinnamon effect in stimulating the secretion of hormones FSH, LH, Estradiol, the start of growth and development of follicles in the ovary is under the influence of these hormones \[^{24}\], while the growing follicle itself secretes estrogen which requires both FSH & LH so that the increase production of FSH & LH requires an increase in their receptors in granular and thecal cell layers which makes more sensitivity of FSH to the increased proliferation and increased secretion of estrogen hormone \[^{21}\], so that the appearance of preantral and antral follicles is due to the increase in the levels of hormones FSH and LH that secreted from of pituitary-front \[^{20}\] and the appearance of corpus luteum in
the treatment group of a aqueous extract cinnamon bark and continues its absence in the control group indicates the occurrence of the ovarian activity in the treatment group and the occurrence of ovulation and the liberalization of the egg through it, the primary function of the corpus luteum is the secretion of large quantities of the progesterone hormone\textsuperscript{[26]}, and this explains the high level of hormone in the treatment group with a aqueous extract cinnamon bark and this reflects the effective of cinnamon in the ovarian activity\textsuperscript{[22]}.

Conclusions:
We conclude from this study that the aqueous extract cinnamomum bark have the ability to raise the level of hormones LH, FSH that secreted from the pituitary as well as the raise of the level of both Estrogen and Progesterone hormones, and the occurrence of ovulation in addition to the appearance of clear histological changes on the ovary, which reflects its impact on the effectiveness of the ovary hence we advise to conduct this experience on other laboratory animals and longer times.

References:


23- Sanguanpong, U.; Kongkathip, N. and Sombatsiri, K. (2000). Possibility as biorational insecticide of formulated neem oil-based pellet with certain essential oils and volatile substances against the rice weevil Sitophilus
oryzae Central Laboratory and Green House Complex, Kasetsart University, Kampaengsean Campus, Bangkok, Thailand. pp:1060-66.


Evaluation of Tensile Strength and Microhardness of Two Nickel-Chromium Based Casting Alloys with the Addition of Different Ratios Recasted Alloy Using Oxyacetylene Flame Casting Techniques


*Hawler Dental College, Hawler University **Baghdad Dental College, Baghdad University, ***Medical Technical Institute, Foundation Of Technical Education

Abstract:

The objective of this study is to evaluate the tensile strength and microhardness of Ni-Cr alloy by adding two different ratios of recasted alloy (25%,50%) and one controlled group (100% fresh alloy),some technicians in our country modifies the casting of Ni-Cr alloy by adding recasted alloy because of high cost of Ni-Cr and low economical state.

We use two different trade mark of Ni-Cr alloy (wiron99 and max-whiteness). According to ANSI/ADA Specification No. 14 (ISO 6871); 30 specimens were made for tensile testing for each alloy type. Another 30 specimens were made for micro-hardness testing which they were square in shape (6mm for each arm and 1mm thickness). ,after statistical analysis it was found that there were no significant differences between each sub-group for the same alloy type for tensile and micro-hardness testing specimens. Although there were a great significant differences between each alloy type.
Introduction:
Turkoz; (1989) evaluated the mechanical properties, such as tensile strength, compressive strength, surface hardness and the elongation and reduction percentages of the length of the metal samples prepared from first, second and third generation castings of a denture base metal. The results revealed only a proportional decrease of surface hardness of the specimens casted from first, second and third melting. While the other properties of all specimens showed no significant differences between themselves. The author concluded that the denture base metals could be melted and recasted again at three times.

Ayad et al., (1996) showed that the use of recasted metal alloys might adversely affect the marginal integrity of gold alloy complete crowns. Al-Hiyasat and Darmani (2005) studied the effect of recasting on the element release and cytotoxicity of five base metal alloys, two Ni-Cr, Co-Cr and Cu-base alloy. Specimens were casted as following: 100% as received alloy, 50% as-received with 50% recast and 100% recast. Ni-Cr alloy was the least cytotoxic followed by Co-Cr, Ni-Cr-Cu and Cu-base alloy. Reasting of alloys significantly increased their element release and cytotoxic level. The Co-Cr alloy was more adversely affected by the recasting than the Ni-Cr alloy. The Cu content in an alloy increases its cytotoxic level remarkably and its release remarkably increased in proportion with the use of recast alloys, and it was most affected by the recasting process followed by Co and Ni.

Al-Samarrai (2005) showed that the recasting of Co-Cr and Ni-alloys with addition of a as-received alloy in different percentages decrease the Co,Cr, Ni ions release and the recasted Co-Cr alloy showed more corrosion resistance than recasted Ni-Cr alloy.

Material and Method:
Molds and specimens design for tensile and micro-hardness test:
A specimen is fabricated from metal (nickel alloy) according to ADA specification no. 14, for tensile specimens Fig. 1. An impression was made for the pre-fabricated specimen with agar-agar impression material; the agar-agar impression material was softened at 60°C. A duplicating flask was used to duplicate the specimen; a thin layer of dental plaster about 5mm was poured to the bottom of the flask to hold the specimen. The flask filled with the agar-agar impression material till the whole specimen was imbedded in the impression material. The flask placed in a refrigerator for about 3hours, to facilitate setting and hardening of the agar-agar impression material. Once the impression material was set the specimen pulled from the impression and then the impression poured with cold cure acrylic resin. The acrylic specimens were finished with acrylic bur to maintain the original standard dimensions.

The acrylic specimens were flanked in casting ring with phosphate bounded investment, then the moulds put in burn out furnace for about one hour.
at 900 C° until the acrylic specimens were burned out, finally they held in centrifugal machine so that the molten Ni-Cr (with oxyacetylene flame) were injected into the ring. Since the temperature of the flame was unable to measure; but it was the same for all the three groups; because the valves that allow passage of the gases were controlled, instead we measure the time at which each group were molten; and it was as follow: for group I about 5minutes, group2 8minutes and for group3 it was 10minute s. Figure-1

![Figure-1: Tensile specimen](image)

The recasted alloy used once for each group i.e. the sprues from 2nd and 3 rd recasting groups were discarded and not used again.

For micro-hardness specimens a square shape specimen was prepared from acrylic resin its dimensions were 1mm thickness and 6mm for each arm. The standard specimen was duplicated by making impression for the specimen with polysulfide impression material. Finally, the surface of the specimens were highly finished and polished with 500-grit silicone carbide paper under running tap water then finally with pumice to get highly polished mirror like surface. Figure-2

![Figure-2: Micro-hardness specimen](image)

**Grouping of the specimen:**

The ratios of the as-received and recasted alloys for each group type were suggested by a previous study by Muhammad F. Mutlak M.F (2004) [5]

**Tensile specimen**

As illustrated in figure-3, two types of nickel chromium alloy were used; wiron 99 and max whiteness. Each group consists of 3 subgroups of different mixing ratio of as received and recasted alloy as following:

A- 5 specimens made from as-received nickel chromium 100% group 1.
B- 5 specimens made from as-received nickel chromium for 75% and the remaining 25% were recasted nickel chromium alloy group 2.
C- 5 specimens made from as-received nickel chromium for 50% and the
remaining 50% were recasted nickel chromium alloy group 3.

Specimens were tested by Tensile strength tester device that showed in Figure-4.

![Tensile Specimens Diagram]

**Figure-3: Grouping of the tensile specimens by wt% ratios**
Micro hardness specimens:

As illustrated in figures (5, 2) types nickel chromium alloy groups were used wiron99 and max whiteness. Each group consists of 3 subgroups of different mixing ratios of as received and recasted alloy as following:

A- 3 specimens made from as-received nickel chromium 100% group 1.
B- 3 specimens made from as-received nickel chromium for 75% and the remaining 25% were recasted nickel chromium alloy group 2.
C- 3 specimens made from as-received nickel chromium for 50% and the remaining 50% were recasted nickel chromium alloy group 3.

Specimens were tested by Rockwell hardness tester device that showed in figure-6.
Figure-5: Shows grouping of the hardness specimens by wt% ratios
Preparation of the grips for tensile specimens:
An adjustment to the tensile testing machine where needed to hold the tensile specimens, since they are too small to hold by the grips of the machine. Therefore two grips were fabricated from chromium steel burned in a specified furnace to make it very hard (harder than the tested nickel chromium material so as not to affect the readings of the specimen) to adapt the specimen to the tensile testing machine. Each grip was made of two equal halves sliced at the middle to hold the specimen between them securely and a nut size 17 used to close the two grip halves with the specimen between them, and these two grips where held securely by the machine. Figure-7

Figure-7: A: The grips holding the tensile specimen before testing.
B: The grips holding the tensile specimen after testing (after rapture).
Results:

Tensile strength:

The Anova test for tensile strength of as-received and recasted groups of both alloy types are illustrated bellow in (Table-1) and (Table-2).

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 2</th>
<th>Group 1</th>
<th>Group 3</th>
<th>Group 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>1.101</td>
<td>1.00</td>
<td>N.S</td>
<td>4.000</td>
<td>1.101</td>
</tr>
<tr>
<td>4.000</td>
<td>1.101</td>
<td>0.01</td>
<td>S.</td>
<td>4.000</td>
<td>1.101</td>
</tr>
</tbody>
</table>

Table-1: Anova test of the tensile strength of the max whiteness samples

<table>
<thead>
<tr>
<th>Group 3</th>
<th>Group 1</th>
<th>-7.000</th>
<th>2.046</th>
<th>0.015</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 2</td>
<td>-3.66</td>
<td>1.305</td>
<td>0.092</td>
<td>N.S</td>
</tr>
<tr>
<td>Group 1</td>
<td>Group 3</td>
<td>7.000</td>
<td>2.046</td>
<td>0.015</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>3.200</td>
<td>2.046</td>
<td>0.372</td>
<td>N.S</td>
</tr>
<tr>
<td>Group 2</td>
<td>Group 3</td>
<td>3.66</td>
<td>1.305</td>
<td>0.095</td>
<td>N.S</td>
</tr>
<tr>
<td></td>
<td>Group 1</td>
<td>2.33</td>
<td>1.305</td>
<td>0.372</td>
<td>N.S</td>
</tr>
</tbody>
</table>

Table-2: Anova test of tensile strength for wiron99

Micro-hardness:

The Anova test for micro hardness of as – received & recasted groups of both alloy types are illustrated as follows in table-3 and table-4:

<table>
<thead>
<tr>
<th>Mean difference</th>
<th>Std. Error</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.1 G.2</td>
<td>-1.33</td>
<td>1.305</td>
</tr>
<tr>
<td>G.3</td>
<td>-3.66</td>
<td>1.305</td>
</tr>
<tr>
<td>G.2 G.1</td>
<td>1.33</td>
<td>1.305</td>
</tr>
<tr>
<td>G.3</td>
<td>-2.33</td>
<td>1.305</td>
</tr>
<tr>
<td>G.3 G.1</td>
<td>3.66</td>
<td>1.305</td>
</tr>
<tr>
<td>G.2</td>
<td>2.33</td>
<td>1.305</td>
</tr>
</tbody>
</table>

Table-3: Anova test for mean of RHN of max whiteness

<table>
<thead>
<tr>
<th>Mean difference</th>
<th>Std. Error</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>G.1 G.2</td>
<td>-0.66</td>
<td>1.67</td>
</tr>
<tr>
<td>G.3</td>
<td>-5.33</td>
<td>1.67</td>
</tr>
<tr>
<td>G.2 G.1</td>
<td>0.66</td>
<td>1.67</td>
</tr>
<tr>
<td>G.3</td>
<td>-4.66</td>
<td>1.67</td>
</tr>
<tr>
<td>G.3 G.1</td>
<td>5.33</td>
<td>1.67</td>
</tr>
<tr>
<td>G.2</td>
<td>4.66</td>
<td>1.67</td>
</tr>
</tbody>
</table>

Table-4: Anova test for mean of RHN of wiron99
Discussion:
The effect of recasting:
Tensile strength:

For max whiteness metal alloy showed a non-significant difference in the tensile strength between the as-received Ni-Cr metal alloy and (75% as-received Ni-Cr metal alloy + 25% recasted Ni-Cr metal alloy), while a significant difference existed when comparisons were made between (50% as-received Ni-Cr metal alloy and 50% recasted Ni-Cr metal alloy) and the other groups separately the same result was observed for wiron99 metal alloy.

While researches made by Strandman (1976) [6], Sheffick (1993) [7] and Abdul-Munim (1994) [8] showed that carbon content of the metal alloy change markedly with repeated melting. The possible explanation is the accumulative gain of carbon from the casting crucible itself with repeated melting. It is observable in laboratories that repeated use of the crucible would render it thinner. This agrees with Craig and Powers (2002) [9] reported that almost all elements in Ni-Cr metal alloys such as chromium, molybdenum and silicon reacts with carbon to form carbides, thereby changes the properties of the metal alloy. If the carbon content where increased by 0.2% over the desired amount, the metal alloy becomes too hard and brittle and could not be useful for making any of the dental appliances. Conversely, a reduction of 0.2% in the carbon content would reduce the metal alloy's yield and ultimate tensile strengths to such low values that, once again, the metal alloy would not be usable in dentistry.

There were high significant differences between the two metal alloy types (wiron99 and max whiteness). Bridgeport et al. (1993) [10] and Craig and Powers (2002) [9] reported that the possible explanation for this could be due to the difference in the composition of minor metal alloying elements that may more or less affect the strength of the casted metal alloy.

Micro-hardness:

Non-significant differences were observed between the different groups of both alloy types but the alloys in the as-received condition showed lower hardness values than those of the respective alloys after casting. Concerning the casting conditions, the hardness values were higher when the casting procedures performed in a non-controlled atmosphere (flame/air) i.e. blowtorch, because Ni-Cr alloys are more susceptible to contamination by carbon and gas uptake from the atmosphere (like oxygen, hydrogen and nitrogen) than from heat source elements itself.

Leinfelder (1989) [11] found that the oxidation of an alloy elements make it harder. In principle, adhesive oxide formers (typically indium, gallium and tin in precious metal alloys as well as elements such as tantalum and chromium, cobalt, nickel, uranium etc ...) were subjected to increased oxidate. This could be due to the low atomic radius of the carbon atom, which allows its diffusion into the lattice, and the format of an interstit..
Phillips (1991) \cite{12} found that the higher hardness values reached by the metal alloys when casted with the blowtorch (flame/air) were because of the ability of some metal alloying elements (such as chromium, titanium, niobium, silicon, molybdenum) in forming carbides. Covington et al: (1985) \cite{13} stated that the amount of carbon absorbed by the metal alloy could be even higher when the metal alloy is overheated. Baran (1979) \cite{14} reported that the uptake of carbon modifies the metal alloy microstructure and its mechanical properties.

However a high significant difference found between the corresponding groups of both metal alloy types, the explanation for this could be due to the difference in the composition of minor metal alloying elements that may more or less affect the strength of the casted metal alloy. This agrees with Bridgeport et al. (1993)\cite{10} and Craig and Powers (2002)\cite{9}.

Finally, it could be summarize that recasted Ni-Cr metal alloy showed good results concerning measured microhardness property that could be used instead of as-received alloy that is more expensive for fabrication of fixed prosthesis, while recasting in gued decrease the tenst strength of Ni-Cr-alloy in different degrees for different ratios of recasting alloy so it is preferable not to use the recasted alloys for fabrication of dental prosthesis that require flexibility like clasps and metal frame works connectors.

References:
recasting of non-precious and implant materials" A thesis submitted to the college of dentistry, university of Baghdad


Antibacterial activity of Thymus vulgaris and Prunus amygdalus extracts against bacterial wound infection

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Collage of pharmacy, university of AL-Mustansriya

The extract of Thymus vulgaris and Prunus amygdalus was tested for its antibacterial activity against a variety of bacterial strains. The results showed that the extracts had significant antibacterial activity against the tested bacteria, particularly against Klebsiella spp. (22.4%), Escherichia coli (22.4%), Proteus spp. (10.4%), Enterobacter spp. (15%), Staphylococcus aureus (15%), Acinetobacter spp. (3%), and Streptococcus spp. (1.5%).

The extracts were tested by agar-well diffusion method at different concentrations (1:2, 1:4, 1:8, 1:16, 1:32). The results indicated that the highest antibacterial activity was observed against Acinetobacter spp. and Staphylococcus aureus at a concentration of 1:2. The extracts also showed good activity against Klebsiella spp., Enterobacter spp., and Pseudomonas spp., with inhibition zones ranging from 8 to 12 mm. The extracts were also effective against Proteus spp., with inhibition zones ranging from 7 to 10 mm.
Abstract:
Eighty clinical swabs were collected from Patients suffering from wound infection, attending medical city hospital. Sixty seven isolates were diagnosed as causative agents, they were Klebsiella spp. (22.4%), Escherichia coli (22.4%), Pseudomonas spp. (18%), Staphyloccocus aureus (15%), Enterobacter spp (10.4%), Proteus spp (7.5%) Streptococcus spp (3%) and Acinetobacter spp. (1.5%). Eleven antibiotics were used for susceptibility test of drugs. Most of isolates were sensitive to Imipenem in percentage 97% and Amikacin in percentage of 89.5% while most isolate were resistance for many antibiotics like Cefotaxim (89.5%), Tetracyclin (82%), Tobramycin (77.6%), Pipracilin (77.6%) and Gentamycin (77.6%), isolates from gram positive bacteria showed sensitivity (100%) for Vancomycin.

Antimicrobial activity of Thymus vulgaris and prunus amygdalus against higher resistance isolates was estimated, using two folds dilution extracts in agar diffusion technique at concentrations of 1:2, 1:4, 1:8, 1:16, 1:32. Thymus vulgaris has shown anti bacterial activity against Acinetobacter spp., proteus spp. And Klebsiella spp. in all concentration were used. The lowest effect was on E. coli (12 mm, 8 mm) at concentration 1:2, 1:4. No effect was clear on Pseudomonas spp., Staphylococcus aureus and Streptococcus spp.

Hexane extract of Sweet almond extract showed the best effect on Klebsiella spp., Enterobacter spp., Proteus spp. at all concentrations used, while E. coli and pseudomonas spp showed sensitivity concentrations of 1:2, 1:4, 1:8, the activity on Gram positive bacteria :Staphylococcus aureus and Straptococcus spp. was at concentrations of 1:2, 1:4 by measuring inhibition zone which was (8mm,7mm) and (10mm,9mm) respectively.

Keywords: Thymus vulgaris, prunus amygdalus, hexane extract, wound infection.

Introduction:
Pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased [1]. The increasing failure of chemo therapeutics and antibiotics resistance exhibited by pathogenic microbial infectious agents had led to the screening of several medicinal plants for their potential antimicrobial activity [2]. Plants have given western pharmacopoeia have given about 7000 different pharmaceutically important compounds and a number of top selling drugs of modern time, e.g. Quinine, artemisinin, taxol and Camptotheacin [3].
Almond (Prunus amygdalus belong to Rosaceae) is an important food crop, varying in use from local consumption as an edible nut in its natural state to inclusion as a major ingredient in manufactured food products, the nutritional importance of almond fruit is related to its Kernal, which is concentrated as a
source of energy, supplying significant amounts of fats, protein and fiber [4]. Almond also include considerably macronutrients and micronutrients [5], and is a good, source of nutrients associated with heart health such as vit. E mono unsaturated fatty acids, arginine and potassium [6]. Almond has antioxidant activity [7], antibacterial, antifungal activity [8] and Almond is used in cosmetics as skin care products [9].

Thyme (Thymus vulgaris, belong to Lamiaceae), has a very long history of folk use for a wide range of ailments it is an excellent antiseptic and due to the presence of phenolic compounds, thymol and carvacrol [10]. Thyme phytochemicals have been used as antioxidant [11], antibacterial and antifungal [12,13], antiprotozoal [14], and in wound healing [15].

The aim of this study was to determine the antibacterial activity of hexane extract of prunus amygdalus and thymus vulgaris against wound pathogenic bacteria.

Materials and Methods:

Specimen Collection:

Eighty swab of wound infection were collected from patients attended medical city hospital in Baghdad during January till March 2008.

Microorganisms:

The typical microorganisms were obtained after culturing the wound swabs on blood agar & macconkey agar (oxoid). Biochemical test (Indol, Tsi, Urea, Simmon citrate and semi solid manitol), suspected colony (Api 20, Biomereux, France) for gram negative bacteria. Manitol salt agar, coagulase and catalase test for S. aureus with gram stain for all isolates [16].

Antibiotic susceptibility test:

Susceptibility test of the isolate was done using the Kirby-Bauer disk diffusion method by using Mueller – Hinton agar, the agar plate left at room temperature for 1h to allow diffusion of the antibiotics into the agar medium. Plates were incubated at 35 – 37 C° for 24h , zone of growth inhibition was then measured to the nearest millimeter and recorded [17].The antibiotics disks used were Imipenem (Imp), Amikacin (AK), Ciprofloxacin (Cip), Cefotaxim (CTX), pipracillin (Pip), Gentamycin (CN), Tobramycin (Tb), Augmentin (Aug), Tetracyclin (TE), Erythromycin (E) and Vancomycin (VA). Isolates were classified as either resistant or inter medium or sensitive based on definition of the clinical and Laboratory standard Institute of WHO .Resistant and intermediate isolate were grouped together for analysis in this study. An isolate was considered multiresistant if it was resistant to at least three of the antibiotics tested , quality control on the susceptibility disk were prformed using laboratory strains E.coli, P.aeruginosa & S. aureus.
Preparation of extracts:
The seeds samples of Almound & Thyme were collected from herbal shops Almound&thyme seeds were milled by using blender, the resulting powder was kept. The extraction was carried out by Soxhlet extraction method using-hexan as solvent Thirty grams of seeds powder was extracted with 300ml of solvent. By using soxhlet apparatus for 10h at temepatur not exceeding the boiling point of the solvent, then the extract concentrated under vacum at 40-50°C by using a rotary evaporator. The consternations 1:2, 1:4, 1:8, 1:16, 1:32 were made by dissolving extract in DMSO (V/V).

Antibacterial assay:
According to The antibacterial activity was evaluated on multi drug resistant bacteria using agar well diffusion Method, using Mueller–Hinton agar (oxoid) for bacteria were selected from 18-24h, turbidity was visually adjusted to that of 0.5 McFarland turbidity standard (1.5 X 10^8 CFU/ml). The inoculums were swabbed on to the surface of agar plates with sterile cotton swab. Six-millimeter wells were punched in to the agar and filled with half dilution of seeds extracts (1:2, 1:4, 1:8, 1:16, 1:32) V/V by transferred to wells using micropipette, DMSO used as negative control. The plates were kept in the refrigerator for 3-5 mint for diffusion and incubated at 37°C for 24h, inhibition zone was measured in millimeter.

Results and Discussion:
Sixty seven isolates were diagnosed as causative bacteria as shown in (Table-1). The most causative agent was Klebsiella spp. and Escherichia coli in Percentage 22.4% for each one, followed by pseudomonas spp.(18%), S. aureus(15%), Enterobacter spp. (10.4%), Proteus spp. (7.5%), Streptococcus spp.(3%), while the lowest causative agent was Acinetobacter spp. in percentage of 1.5%.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>No.</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>15</td>
<td>22.4</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>15</td>
<td>22.4</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>7</td>
<td>10.4</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>5</td>
<td>7.5</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>Total</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

Table-1: percentage of Bacterial isolate caused wound infection.
Studies showed the following microorganisms act as causative agent of wound infection: S. aureus (17%), Enterococci (13%), coagulase negative Staphylococcus (12%) Escharichia coli (10%), Pseudomonas spp. (8%) Enterobacter spp. (8%) proteus mirabilis (4%), Klebsiella pneumonia (3%), and Candida albicans in percentage 2% [22,23].

The sensitivity of isolated bacteria to antibiotics was shown in (Table-2) the more effective antibiotic was Imipenem, the isolates showed sensitivity in percentage of 97% followed by Amikacin, Ciprofloxacin, pipracyllin, Gentamycin, Augmentin, Tetracyclin, tobramycin, Erythromycin and cefotaxin.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>IMP (10) mg</th>
<th>AK (15) mg</th>
<th>CIP (10) mg</th>
<th>PIP (100) mg</th>
<th>CN (30) mg</th>
<th>Au (10) mg</th>
<th>TE (30) mg</th>
<th>TB (10) mg</th>
<th>CTX (10) mg</th>
<th>E (30) mg</th>
<th>V (25) mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>13</td>
<td>12</td>
<td>13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>11</td>
<td>12</td>
<td>11</td>
<td>13</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>9</td>
<td>11</td>
<td>11</td>
<td>9</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acinetobacter spp</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total of Resistant isolates</td>
<td>2 (3%)</td>
<td>7 (10.4%)</td>
<td>30 (44.8%)</td>
<td>52 (77.6%)</td>
<td>52 (77.6%)</td>
<td>52 (77.6%)</td>
<td>55 (82%)</td>
<td>55 (82%)</td>
<td>60 (89.5%)</td>
<td>6 (50%)</td>
<td>0 (6%)</td>
</tr>
</tbody>
</table>

Table-2: Number and Percentage of Resistant bacterial isolates.

The antimicrobial properties of planties of plants have been investigated by a number of researchers worldwide [24]. The nature and number active antibacterial principles involved in Thymus vulgaris and Prunus amygdalus extracts of our study are not investigated but the results showed the antibacterial activity of those extracts against multi drug resistant pathogenic bacteria, as shown in (Table-3) and (Table-4).

The results of antibacterial activity of Thymus vulgaris seeds extracts in concentrations of 1:2, 1:4, 1:8, 1:16, 1:32 were shown in (Table-3). The highest effect was on Proteus spp., Klebsilla spp. and Acinetobacter spp. at all concentrations used with different inhibition zone diameters. Enterobacter spp. appeared inhibition zones 13mm, 12mm, 11mm, 8mm at concentrations of 1:2, 1:4, 1:8, 1:16 respectively. The lowest inhibition zone was on Escherichia coli.
which was 12mm, 8mm at concentrations of 1:2, 1:4, respectively. No inhibition was against *Pseudomonas* spp. and Gram positive bacteria: *S. aureus* and *Streptococcus* spp.

The strongly antiseptic and antifungal activity of thyme is mainly due to the presence of phenolic compounds, thymol and carvacrol (10), the content of thymol in thyme essential oil is much higher compared to carvaerol content [25].

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Inhibition zone (mm) in diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>12</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>13</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>13</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>13</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>-</td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>13</td>
</tr>
</tbody>
</table>

**Table -3: Antibacterial activity of hexane extract of *Thymus valyaris* seeds.**

(Table-4) showed the antibacterial activity of hexane extract of *prunus amygdalus* on isolated multidrug resistant bacteria, *Klebsiella* spp., *Proteus* spp. and *Enterobacter* spp. showed sensitivity to all concentrations used with differences in inhibition zone diameters. *Escherichia coli*, *Pseudomonas* spp. and *Acinetobacter* spp. were sensitive in concentrations 1:2, 1:4, 1:8, almond extract have shown activity against Gram positive bacteria, The highest effective was on *Streptococcus* spp. It was 10mm, 9mm at concentration of 1:2, 1:4 respectively while Inhibition zone was 8mm, 7mm for *S. aureus*. The activity of almond maybe due to its content of oils [21], flavonoids and phenols [6].

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>Inhibition zone (mm) in diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>9</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>15</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>12</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>8</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>13</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>12</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>10</td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>14</td>
</tr>
</tbody>
</table>

**Table-4: Antibacterial activity of hexane extracts of *prunus amygdalus* seeds.**
The mechanisms of action of antimicrobial effects is due to active principles which toxic to microbial pathogens or they may be impair variety of enzyme systems including those involved in energy production and structural component synthesis. Conclusively, plants are evaluable sources for new compounds and should receive special attention in research strategies to develop new antimicrobial agents.

References:
6- Chen, Cy; Mibury, PE; Lapsley, K. and Blumberg, JB. (2005). Flavonoids from Almond Skins Are Bioavailable and act Synergistically with vitamin C and E to Enhance Hamster and Human LDL Resistance tooxidation the American Society for Nutritional Science 135:1366–1373.


