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Formulation and Clinical Evaluation of Orphenadrine citrate as a Plain Tablet

Abdul Karim F. Jumaa, Alaa A. Abdurrasool and Hikmet A. Al – Dujali

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ABSTRACT

Orphenadrine is an anticholinergic, antimuscarinic, centrally acting skeletal muscle relaxant. It presents in the form of citrate and HCl salts which are used in treatment of the symptoms of mild Parkinson's disease and also it is used as adjuvant with other drugs in the therapy.

Many trials were made to formulate Orphenadrine citrate as a plain tablet using wet granulation or direct compression technique in order to get a satisfactory formula through studying the effect of various factors such as binders, diluents and disintegrants types.

The best formula was obtained by using Poly Vinyl Pyrolidine (PVP) as a binder also the results indicated that starch and mannitol gave acceptable physical properties to the tablets when they were used as diluents. At the same time, the results showed that Avicel which was used as a disintegrant gave an acceptable disintegration and dissolution time in comparison with the reference tablet DISIPAL®. In addition, the selected formula was used to study the effect of method of incorporation of disintegrant on the physical properties of tablets. It was found that the intragranular incorporation resulted in a shorter disintegration and dissolution times. The stability of Orphenadrine citrate prepared tablets was also studied upon storage at 50°C, 60°C and 70°C for four months. The drug was fairly stable and the expiration date for the prepared tablet was considered to be equal for 5 years. On the other hand, the results of clinical study on patients suffering from Parkinson's disease indicated that patients with tremor (regular rhythmic oscillation of extremities especially hand and finger) and mild symptoms of Parkinson's disease showed a good response to the prepared tablets, but it had no effect on patients of dystonia (fixed upward gaze, neck twisting, clenching jaws) and akinesia (slow down of movement of voluntary muscle and difficulty of initiation of movement).

The overall results of this study indicate that the drug can be prepared as tablets, which fit the requirements of British Pharmacopoeia since the prepared tablets gave satisfactory results.

伊拉克药学院，大学药学部，巴格达大学，巴格达-伊拉克
INTRODUCTION

The skeletal muscle relaxants are group of compounds used to relieve spasticity & abnormally high muscle tone \(^{(12)}\). They produce their effects by action on central nervous system (CNS) however, their mechanism of action not yet understood. There are many theories, which explain the mechanism of action or the clinical uses of muscle relaxants. One of these theories is that they reduce skeletal muscle spasm, possibly through an atropine like central action on cerebral motor centers or on the medulla but they do not have analgesic activity that contribute to their effects in patients with skeletal muscle spasm \(^{(5)}\). Anticholinergic drugs (Antimuscarinic drugs) were the most effective drugs for treatment of Parkinson's disease for more than a century, this is by blocking Acetyl choline receptors of the CNS, there by partially redressing the imbalance created by decreasing dopaminergic activity \(^{(4)}\). However, the introduction of the dopaminergic drugs (Levodopa & decarboxylase inhibitors) has relegated anticholinergics to a supportive role in the treatment of the disorder.

Nevertheless, the anticholinergic drugs are still useful for patients with minimal symptoms as patients unable to tolerate levodopa because of side effects or contraindications, for those who are not benefited by levodopa \(^{(5)}\) & for patients who had parkinsonian symptoms induced by antipsychotic drugs \(^{(6)}\). \(^{(7)}\). Orphenadrine citrate is white or almost white, odorless or almost odorless, crystalline powder with a bitter taste & followed by sensation of numbness. It melts in the range of 134 °C to 138 °C \(^{(8)}\).

Soluble 1 in 70 of water, slightly soluble in ethanol; practically insoluble in chloroform and ether \(^{(9)}\). It should be stored in tight & light resistant containers \(^{(10)}\).

\[
\begin{align*}
\text{CH}_2 - \text{COOH} \\
\text{m} & \text{E} & \text{C} & \text{N} & \text{Me} & \text{C} & \text{HO} & \text{C} & \text{COOH} \\
\text{CH}_2 - \text{COOH}
\end{align*}
\]

N,N dimityl [2 – (2 – methylbenzyldoxy) ethyl] amine dihyd-rogen citrate \(^{(11)}\), \(\text{C}_9\text{H}_9\text{NO}_3\text{C}_6\text{H}_2\text{O}_7\) with M.wt of 461.5 gm.

Orphenadrine citrate is used for symptomatic treatment of Parkinson's disease \(^{(11)}\) to relieve pain due to spasm of voluntary muscle \(^{(12)}\) and as an alternative to quinon in treatment of noctural leg cramps \(^{(13)}\). Orphenadrine may also be used in vertigo in patient with spontaneous vestibular disease \(^{(14)}\), and it may be combined with haloperidol in treatment of chronic schizophrenic patient \(^{(15)}\) or with paracetamol in treatment of Myalgia \(^{(14)}\).

Orphenadrine is contraindicated in patient with glaucoma \(^{(16)}\), elderly people \(^{(18)}\) and with antacid \(^{(19)}\). Its overdose is treated with physostigmine \(^{(20)}\) or tetrahydroaminocrine \(^{(21)}\).

This study was carried out to formulate Orphenadrine citrate as a tablet dosage form, through preparing different formulas, and comparing them with reference tablets. Also the effect of excipients type (binders, disintegrants and diluents) on physical properties of the tablet was studied in addition to the effect of incorporation method of disintegrants.

Furthermore, the selected formula, which fitted the standard requirements, was thoroughly investigated for its expiration date and clinical effects.

**EXPERIMENTAL PART:**

- Orphenadrine citrate powder, (Dar AL Dawa, Amman, Jordan), Starch, Mannitol (Merk, Darmstadt, Germany), Microcrystalline cellulose (Avicel PH101, FMC corporation, Pennsylvania, USA), Polyvinyl pyrolidine (PVP, K30), Ethanol, Hydrochloric Acid (HCl), Carboxy methylcellulose sodium salt (CMC), Isopropyl alcohol, (BDH Chemicals Ltd., pool, England), Magnesium stearate, (Barloches, GMBH, Germany), Acacia arabique, Dextrose, Talc, (Riedel – De – HAEN AG Seez – Hannover, Germany), Dibasic calcium phosphate (Emcompress, Edward Mendell Co., USA), Explotab (AVEBA, Veendom, Netherlands), DISIPAL tablets as a reference (Yamanouchi Pharma Ltd., UK).
Formulation of Orphenadrine citrate Tablets

Different formulas (Table I) were prepared to find the most satisfactory formula using wet granulation technique except formula 7 which was prepared by direct compression technique, in which the drug and excipients (except lubricant) were dry blended for at least 5 minutes and then mixture was compressed into tablets using F3 Manesty tablet machine with a single 7 mm normal concave punches.

In case of using wet granulation method, the following procedure was followed: after 5 minutes dry blending of drug & excipients, the binder solution was added to the formula gradually in the mixing mortar until a satisfactory wetting was achieved (Ball test). The wet mass was then granulated through a sieve no. 10 and dried in a tray oven at 45°C for 30 minutes. The granules were then reduced in size and homogenized by passing them through a sieve no.16.

A known weight of the granules was then mixed with specified amount of disintegrant extra – granularly for 10 minutes in well closed container and then mixed with magnesium stearate (200 mesh in size) for 2 minutes. The final mixture was compressed.

Physical Parameter Measurement of Orphenadrine Tablets

Hardness: The hardness of Orphenadrine citrate tablets were measured using Monsanto and Erweka hardness testers normal range between 4& 8 kg(20).

Weight Variation: It was determined for all prepared formulas by taking 20 tablets, weighed individually and the average weight is calculated. For the tablets to be acceptable by not more than 2 of the 20 tablets may differ from the average weight by not more than 7.5% and no tablet may differ by more than double the percent (25).

Friability Test: The friability of tablets was performed using Roche friabilator and Erweka fribrilator for 4 minutes at 25 r.p.m by weighing 10 tablets then place them inside the tester for 4 minutes and weigh them again. The difference in weight should not exceed 1%.

Disintegration Time: The disintegration time was measured using U.S.P disintegration apparatus. It consists of a basket rack assembly containing six open –ended glass tubes with a 10 –mesh screen on the bottom. The basket was immersed in an appropriate fluid (0.1N HCl ) at 37 °C. The basket rack was raised and lowered at a rate of 30 stockes per minute (25).

Table (1)

<table>
<thead>
<tr>
<th>Materials</th>
<th>Formula No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Orphenadrine citrate (mg)</td>
<td>75</td>
</tr>
<tr>
<td>PVP10%w/v in ethanol</td>
<td>Q.S</td>
</tr>
<tr>
<td>Avicel pH101</td>
<td>X</td>
</tr>
<tr>
<td>Starch</td>
<td>Y</td>
</tr>
<tr>
<td>Mg.stearate</td>
<td>1.5</td>
</tr>
<tr>
<td>Dextrose</td>
<td>D</td>
</tr>
<tr>
<td>Acacia 20%</td>
<td>Q.S</td>
</tr>
<tr>
<td>Mannitol</td>
<td>M</td>
</tr>
<tr>
<td>Explotab</td>
<td>E</td>
</tr>
<tr>
<td>Starch (disintegrant)</td>
<td></td>
</tr>
<tr>
<td>Emcompress</td>
<td></td>
</tr>
<tr>
<td>Starch paste</td>
<td></td>
</tr>
<tr>
<td>CMC</td>
<td></td>
</tr>
<tr>
<td>Total weight</td>
<td>150</td>
</tr>
</tbody>
</table>

Q.S = sufficient quantity, X= amount of Avicel , Y= amount of Starch , D= amount of Dextrose , M= amount of Mannitol , E= amount of Explotab , S= amount of Starch (disintegrant) .
Dissolution Test: The USP Basket Method\(^{(26)}\) was used to study the release of the drug from the prepared tablets and from the reference tablet DISIPAL\(^{®}\) (Yamanouchi). The studies were carried out in shielded apparatus to protect the solution of drug from light using 900 ml of 0.1N HCl solution as the dissolution medium at 37\(^{°}\)C with a constant stirring speed of 50 r.p.m\(^{(26,27)}\). Samples were withdrawn at five minute intervals for one hour. The sample volume was replaced immediately by a fresh 0.1N HCl. The samples were filtered by microfilter and analyzed spectrophotometrically at its \(\lambda_{\text{max}}\) 264 nm\(^{(28)}\).

Assay for total Orphenadrine citrate present in the Tablets:
Preparation of standard: 150 mg of pure Orphenadrine citrate was dissolved in 75 ml 0.1N HCl, shaken for 15 minutes and filtered, then the volume was completed to 100 ml with 0.1N HCl. A sample of 20 ml was taken and diluted to 100 ml with 0.1N HCl. The absorbance of the diluted solution was determined spectrophotometrically at its \(\lambda_{\text{max}}\) which is 264 nm\(^{(28)}\).

Assay of the Prepared Tablets: 20 tablets of Orphenadrine citrate were triturated and an accurate weight equivalent to 0.15 gm of Orphenadrine citrate was added to 75 ml of 0.1N HCl, shaken for 15 minutes and filtered. The final volume was completed to 100 ml with 0.1N HCl.

The absorbance of diluted solution was determined spectrophotometrically at 264 nm, and the quantity of drug per tablet was calculated according to the following equation:

\[ \text{Test} / \text{Std.} \times 100 = \% \text{ Orphenadrine citrate present in tablet}\(^{(28)}\).

Kinetic Study:
Effect of temperature on Orphenadrine citrate tablets was studied by storing some tablets of the selected formula (1) at different temperatures (50 °C, 60 °C, 70 °C) for four months. Samples of tablets were taken at desired time intervals and assayed for contents of Orphenadrine citrate according to the method mentioned before. The friability, disintegration and dissolution time were also checked at the end of four months, and organoleptic properties were also examined.

Statistical Analysis:
Students t – test was used to examine the difference in the mean of the results of parameters tested. A p – value of less than 0.05 was considered significant.

Preliminary Clinical Study:
The selected prepared formula (1) in addition to DISIPAL\(^{®}\) were given to seven patients suffering from tremor (legs and arm ), stiffness and autonomic dysfunction which are clinical symptoms of Parkinson's disease. They were also given to three patients with dystonia, at the same time the study was done using placebo tablets. The treatment was followed up to three months with a dose of one tablet 3 times daily. The study was done in Nahrain College of Medicine Teaching Hospital to determine the clinical symptoms and the clinical parameters used to assess the therapy.

Results and Discussion:
Effect of Binder Type:
Different formulas (1, 3, 8 and 9) were prepared to study the effect of binder type on the hardness, friability, disintegration and dissolution times as shown in table (2) and fig.

<table>
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<th>Suggested formula</th>
<th>Type of binder</th>
<th>Hardness (kg)</th>
<th>Friability (%)</th>
<th>Disintegration Time(min)</th>
<th>Dissolution Time(min) to 100%</th>
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</thead>
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<tr>
<td>1</td>
<td>10% PVP</td>
<td>8.25</td>
<td>0.539</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>20% Acacia Mucilage</td>
<td>7</td>
<td>0.7</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>10% Starch paste</td>
<td>5</td>
<td>1.1</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>9</td>
<td>5% CMC</td>
<td>3</td>
<td>2.8</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>DISIPAL(^{®}) Reference</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table (2)
Effect of Binder Type on the hardness, friability, disintegration time and dissolution time of the suggested Orphenadrine Citrate formula in comparison with the reference tablet.
The results obtained indicate that using different types of binders affect the physical properties and the release of drug. For example, formula 1 in which PVP was used as a binder, showed reasonable hardness (8.25kg), friability (0.5%), disintegration time (5 mins.) and dissolution time up to 100% (20 mins.) in comparison with the reference tablet DISIPAL \(^6\). This may be due to the property of starch paste , which forms generally soft and brittle tablets\(^{31}\). Finally formula 9 in which CMC sodium is viscosity controller, it is conceivable that it forms a highly viscous system that resists dilution by dissolution fluid which might impede drug release\(^{32}\).

**Effect of Diluent Type**

Formulas 1, 2, 4 and 7 were used to study the effect of diluent type on the hardness, friability, disintegration and dissolution times of the prepared tablets.

Table (3) shows the effect of diluent type on physical properties of the prepared tablets, while fig (2) shows the release of Orphenadrine citrate from the prepared tablets (formula 1, 2, 4 and 7) in comparison with the reference tablets DISIPAL \(^6\).

Formula 1 in which starch was used as diluent gave a good hardness (8.25kg), friability (0.539%), disintegration time (5 mins.) and dissolution time up to 100% (20 mins.).

Formula 2 which contains dextrose as a diluent, showed a good friability (0.1%), hardness (7kg) and disintegration time (4 mins.) but long dissolution time (40 mins.), although brown spots was seen on the tablet after a while which may be due to the interaction of dextrose with amines (Orphenadrine)\(^{38}\). Formula 4 in which mannitol was used as a diluent, gave a good friability (0.5%), hardness (7.25kg), disintegration time (6 mins.), and dissolution time up to 100% (20 mins.).

Formula 5, which was prepared by direct compression technique, showed unacceptable friability (1.1%) and hardness (3kg) with a rapid disintegration time (50 secs.) and dissolution time of 20 min.

**Effect of Disintegrant**

Formulas 1, 5 and 6 were utilized to study the effect of disintegrant type on physical properties of the prepared tablets as shown in table (4) and fig (3).
Table (3)

Effect of Diluent on the Physical Properties of Orphenadrine Citrate Plain Tablets

<table>
<thead>
<tr>
<th>Formula No.</th>
<th>Diluent Type</th>
<th>Hardness (kg)</th>
<th>Friability (%)</th>
<th>Disintegration time (min)</th>
<th>Dissolution Time (min) to 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Starch</td>
<td>8.25</td>
<td>0.539</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Dextrose</td>
<td>7</td>
<td>0.1</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>Mannitol</td>
<td>7.5</td>
<td>0.5</td>
<td>6</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>Emcompress</td>
<td>3</td>
<td>1.1</td>
<td>0.5</td>
<td>20</td>
</tr>
</tbody>
</table>

Table (4)

Effect of Disintegrant Type on the Physical Properties of Prepared Orphenadrine Tablets

<table>
<thead>
<tr>
<th>Formula No.</th>
<th>Diluent Type</th>
<th>Hardness (kg)</th>
<th>Friability (%)</th>
<th>Disintegration time (min)</th>
<th>Dissolution Time (min) to 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Avicel</td>
<td>8.25</td>
<td>0.539</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Explotab</td>
<td>4</td>
<td>0.1</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Starch</td>
<td>3.5</td>
<td>0.01</td>
<td>4</td>
<td>30</td>
</tr>
</tbody>
</table>

Fig. 3: The effect of disintegrants type on the release of Orphenadrine in comparison with the reference tablets DISIPAL®

Formula 5, in which Explotab (low substituted carboxymethyl starch) was used as a disintegrant showed a relatively low hardness (4kg) with an acceptable friability (0.1%), disintegration time (5mins.) and dissolution time (25 mins.), however, its dissolution profile showed no significant difference with formula 6 (p-value > 0.05).

This may be due to Explotab properties since its granules absorb water rapidly and swell but do not break. In general, the swelling granules remain intact, causing disintegration without bursting (unlike starch) and consequently release of the soluble starch fraction. This might lead to increase the viscosity and delay moisture penetration into the tablet.

Formula 6 showed a good friability (0.01%), disintegration time (4mins.) and dissolution time (30mins.), with relatively low hardness (3.5kg). This may be due to the fact that tablets containing high amount or concentration of starch are often soft and may be difficult to dry. Formula 1 in which Avicel® was used as disintegrant, showed a good hardness (8.25kg) and friability (0.5%) with an acceptable disintegration time (5mins.) and a fast dissolution time (20mins.).

This is because Avicel® is a super disintegrant which is highly porous with strong "wicking" tendencies, this will allow water to enter the tablets matrix by means of capillary forces which breaks the hydrogen bonding between adjacent bundles of cellulose microcrystals.

Effect of Mode of Incorporation of Disintegrant

Formula 1, was used to study the effect of method of incorporation of disintegrant. It was prepared by three methods of incorporation, they were: extragranular 1, intragranular 1b, and combination of both types 1c. The data are displayed in fig (4) and table (5).
A Comparison Between the Effect of Method of Incorporation of Disintegrant Extra or Intra or Combination of Both

<table>
<thead>
<tr>
<th>Formula No.</th>
<th>Disintegrant Location</th>
<th>Hardness (kg)</th>
<th>Friability(%)</th>
<th>Disintegration Time (min)</th>
<th>Dissolution Time (min) to 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Avicel Extra</td>
<td>8.25</td>
<td>0.539</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>b</td>
<td>Avicel intra</td>
<td>3</td>
<td>0.1</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>c</td>
<td>Avicel Extra &amp; intra</td>
<td>3.5</td>
<td>0.15</td>
<td>4</td>
<td>15</td>
</tr>
</tbody>
</table>

The results showed that faster disintegration (3 mins.) and shorter dissolution time (10 mins.) for formula 1b compared with that of formula 1c and formula 1. This is in agreement with Gordon et al. (34) who stated that incorporation of super disintegrant in the intragranular phase resulted in faster tablet dissolution than did incorporating it in the extragranular phase or both phases. Based on the overall results; it seems that formula 1 and 5 are the promising formulas compared with the reference tablet DISPAL. Since both of them showed good disintegration and dissolution times. However, regarding the economic part of production and the cost of mannitol (33), formula 5 was excluded.

Kinetic Study

The stability of formula 1 which was chosen as the promising formula was studied at different temperatures (50, 60 and 70 °C) for four months. The degradation of Orphenadrine citrate follows first order kinetic since straight lines were obtained when the logarithm of percent remaining of Orphenadrine citrate was plotted versus time (fig. 5). The degradation rate constants (K) for different temperatures were calculated from the slopes of the lines as shown in table (6).

Table 6 Rate Constants of Degradation (K) of Orphenadrine Citrate (formula 1) at Different Temperatures

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>K(month⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.0×10⁻³</td>
</tr>
<tr>
<td>60</td>
<td>1.413×10⁻³</td>
</tr>
<tr>
<td>70</td>
<td>1.995×10⁻³</td>
</tr>
</tbody>
</table>

Fig 4: Effect of mode of incorporation of disintegrant on the release of Orphenadrine from prepared formulas

Fig 5: Accelerated breakdown of Orphenadrine citrate at different elevated temperatures (formula 1)
To compute the expected expiration date ($t_{10\%}$), Arrhenius plot was made to predict the $K_{25^\circ C}$ as shown in fig (6), utilizing the following equation:

$$0.104 = -\frac{t_{10\%}}{K_{25^\circ C}}$$

However, the calculated $t_{10\%}$ was long, so it is considered to be equal for 5 years. In addition, at the end of four months, no change in physical properties of the prepared tablets was seen.

**RELIMINARY CLINICAL STUDY**

The results of this study as indicated in table (7) showed five out of seven patients had a good response to the drug after one week of treatment for the selected formula 1 as well as the reference tablets DISPAL®, while the rest two patients showed different behavior, i.e., one had no response to the drug and the other showed side effects such as hallucination and blurred vision after one day of treatment, therefore the therapy was stopped, on the other hand, the drug showed no effect on patients with dystonia as with the placebo tablets.

**CONCLUSION**

From all previous experimental work one can conclude that best binder is PVP in ethanol since it is cheap, available, compressible and compatible with drug. Starch and Mannitol are the best diluents but we prefer using starch because of its low cost. Avicel is a good disintegrant since it is compressible, highly porous giving a good disintegrating time although it is relatively expensive. Formula 1 is the most satisfactory formula in comparison with the reference tablets DISPAL®, and the $t_{10\%}$ was considered to be equal for 5 years.

Table (7)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (year)</th>
<th>Sex</th>
<th>Chief complaint</th>
<th>Past treatment</th>
<th>Duration new treatment</th>
<th>Dose</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>Male</td>
<td>Tremor</td>
<td>Parkizol sinamet</td>
<td>One day</td>
<td>1×3</td>
<td>Patient develop hallucination and blurred vision stop treatment after one dose only</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>Male</td>
<td>Tremor</td>
<td>No</td>
<td>2 weeks</td>
<td>1×3</td>
<td>Moderate response</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>Female</td>
<td>Tremor</td>
<td>Sinamet</td>
<td>2 months</td>
<td>1×3</td>
<td>Good response</td>
</tr>
<tr>
<td>4</td>
<td>59</td>
<td>Male</td>
<td>Tremor</td>
<td>No</td>
<td>3 months</td>
<td>1×3</td>
<td>Good response</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>Female</td>
<td>Tremor</td>
<td>No</td>
<td>1 week</td>
<td>1×3</td>
<td>No response</td>
</tr>
<tr>
<td>6</td>
<td>40</td>
<td>Female</td>
<td>Tremor and rigidity</td>
<td>Parkizol Sinamet artane</td>
<td>1 month</td>
<td>1×3</td>
<td>Good response</td>
</tr>
<tr>
<td>7</td>
<td>45</td>
<td>Male</td>
<td>Tremor</td>
<td>No</td>
<td>2 weeks</td>
<td>1×3</td>
<td>Moderate response</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>Female</td>
<td>Dystonia</td>
<td>No</td>
<td>3 weeks</td>
<td>1×3</td>
<td>No response</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>Male</td>
<td>Dystonia</td>
<td>No</td>
<td>1 month</td>
<td>1×3</td>
<td>No response</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>Male</td>
<td>Dystonia</td>
<td>No</td>
<td>2 weeks</td>
<td>1×3</td>
<td>No response</td>
</tr>
</tbody>
</table>

% response to Tremor 71.4
% side effect 14.28
% response to dystonia 0.0

Note: The difference between good & moderate responses is related to the examiner.
REFERENCES
1. C.W.T Pilcher ; Parkinsonism ; Kuwait drug index ; Kuwait drug index committee;1988,1st edition ; 441 – 449.
32. Swinyard E.A. ; Lowenthal W. ; Carboxy methyl cellulose sodium ; emulsifying and suspending agents ; pharmaceutical Necessities in Remington's pharmaceutical Sciences ; 17th edition ; Mack publishing Company ; 1985 , P 1297.
33. Banker G.S.; Rhodes C.T ; Bioavailalability in modern pharmaceutics , Marcel Dekker , New York and Basel ; 1979 , Vol .7;P143 .
A Study of the Furocoumarin Derivative of Ruta Chalepensis L. (Rutaceae)
Ekal H. AL-Khateeb, Ali A. AL-Shamma, Bajes A. Nehar
Received 2-3-2002 Accepted 23-10-2002
ABSTRACT
The content of Furocoumarin derivatives (Psoralens) of Ruta Chalepensis L. (Whole plant) was studied by simple extraction with petroleum ether (b.p. 60-80°C). The results indicated that the plant contains a total of about (0.015%).

Investigation of these compounds by thin layer chromatography (TLC) revealed the presence of at least four compounds of which methoxsalen (8-methoxypsoralen) was isolated. It was identified and authenticated with a standard by spectral method, IR, NMR, Mass spectra and HPLC. This plant could be considered as a good source for supplying this compound, which is widely used in dermatological preparations.

INTRODUCTION
Ruta Chalepensis L. (Rutaceae) is a cultivate plant[1] widely grown as garden ornamentals in Baghdad and its vicinity. Ruta, Rue and Sadhab are common names for this plant. Rutaceae (also termed Rue family) is one of the important families, that comprises many genera and species which have been used as a source of drugs and medicines[2]. The decoction of this plant has been used in folk remedy as carminative to cure stomach ache in children[3].

This study was carried in connection with a program of using the chemical constitutes of Iraqi Medicinal Plants as a potential for industrial purposes. Literature survey revealed that Ruta chalepensis L., contains many important components[4], among which are coumarins and Furocoumarin derivatives. Since no previous work has been done on this Iraqi plant was found that study of these compounds, is of advantage for its economical value and uses in dermatological preparations, as available imported ones are very expensive.

MATERIALS and METHOD
The plant material was collected during September and October 1997 from the gardens of the vicinity of the city of Baghdad. Four and a half kilograms of powdered plant material (aerial plants and roots) were extracted with 12 liters of petroleum ether (b.p. 60-80°C), under reflux for 72 hours. The petroleum ether extract was filtered and evaporated to dryness, under reduced pressure at a temperature not exceeding 40°C, leaving a dark green residue (80 gm).
Separation and Fractionation of Furocoumarin Derivatives:

Different partition processes together with column chromatography technique were tried but with unsuccessful results. So the separation and fractionation was carried out by using preparative liquid layer chromatography.

A quantity of 20 gm of the residue was dissolved in a minimum amount of chloroform and applied together with standard psoralen reference compounds (methoxsalen and imperatrin) on a number of preparative TLC plates of silica gel GF254 (0.75 mm thickness) and developed with solvent system benzene-acetone (90:10 v/v).

The developed plates were visualized under U.V light (366 nm wavelength). Four major bands were observed and designated as band I, II, III and IV.

Band II and III were overlapped, and both have very close Rf values to that of the standard reference methoxsalen.

These two bands were separated by multiple development using the same solvent system to give two compounds.

Band I gave compound I as yellowish crystals (25 mg) upon recrystallization out of boiling methanol, a fluffy colorless crystals was obtained (20 mg) having a sharp melting point of 147.5°C. Out of band II, compound 2 was isolated as yellowish prismatic crystals (125 mg), upon recrystallization out of hot petroleum ether (b.p. 60°C) a colorless crystals (25 mg) upon recrystallization out of boiling methanol, a fluffy colorless crystals was obtained having a sharp melting point of 78°C.

Out of band III, compound 3 was isolated as a yellowish crystals (2.7 mg) upon recrystallization out of boiling ethanol fluffy like crystals (2.5 mg) was obtained. Out of band IV, compound 4 was isolated as an oily material. The total yields of these compounds isolated from the total extract (80 gm) were: 80 mg of compound 1; 480 mg of compound 2; 10 mg of compound 3; and oily material designated as compound 4.

RESULTS and DISCUSSION

Out of these compounds, only compound 1 was identified and confirmed to be methoxsalen (xanthotoxin) as it has similar melting point, CH analysis, and Rf values in different solvent systems with standard reference of methoxsalen.

An assay by HPLC also was done for compound 1, using methoxsalen U.S.P. 1% as a standard reference. The retention time was exactly the same, further identification was carried out by enrichment technique, in which the reference was added individually to compound 1, using three different mobile phases (Fig. 1a, b, c).

Other spectral methods were used for further confirmation. The mass spectrum of compound 1 indicates a molecular ion peak at m/e 216. The fragmentation pattern showing significant peaks as shown in (Fig. 2). The NMR spectrum in CDCl3 (Fig. 3) confirmed the presence of eight protons. The proton (H3) and (H4) as two doublet at δ 6.8 (H3) and δ 7.71 (H4) (shielded (J) is small as expected for the furor ring (J=1-2 Cps)). (H5) proton showed singlet peak at δ 7.82 (aromatic proton (H5) and (H6) protons also appeared as two doublet peaks at δ 7.35, δ 7.26 for (H5) proton, at δ 6.43 and δ 6.31 for (H3) proton. Methoxy group protons showed at δ 4.3. The IR spectrum (KBr) (Fig. 4) confirms the structure. C = O stretching band at 1575 cm⁻¹ for the α₁ β-unsaturated lactone. Two bands at 1600 cm⁻¹ 1575 cm⁻¹ for (C = C) stretching vibration of the aromatic ring. (C = C) olefinic band at 1660 cm⁻¹. Two bands at 1450 cm⁻¹ and 1375 cm⁻¹ for asymmetric bending vibration of CH₃ group respectively. The stretching (C-O-CH₂) appears at 1280 cm⁻¹ for asymmetrical stretching and 1020 cm⁻¹ for symmetrical stretching. Two characteristic stretching vibrations for the ester function appear at 1145 cm⁻¹ and 1090 cm⁻¹. The most characteristic out of plane C-H bending vibration at 822 cm⁻¹ and 760 cm⁻¹. Moreover the C-H out plane bending vibration at 870 cm⁻¹ is due to one free (H) in benzene ring. Finally the out of plane olefinic (H) (cisolefein) is present at 755 cm⁻¹ in agreement with the structure.

It is mentioned spectral data confirmed that compound 1 is methoxsalen having the following structure:

![Compound](image)
Fig (1a) H.P.L.C for a mixture of equal quantities of the compound (BA₁) and the standard reference Oxsoralen : (Methoxsalen U.S.P 1%) with second mobile phase.

Fig (1b) HPLC for the compound (BA₁) compared to the standard reference oxysoralen ; (Methoxsalen U.S.P 1%)
Fig.(1c) HPLC for a mixture of equal quantities of the compound (BA₁) and the standard reference Oxssalen; (Methoxsalen C.S.P 1%) with third mobile phase

Fig (2) Mass Spectrum of the compound (BA₁)
Fig (3): NMR Spectrum of the compound (Ba) determined in CDCl₃.

Fig (4): IR Spectrum of compound (BA1)
REFERENCES

The Ability of Nutrient Antioxidants to Influence Oxidative Stress and Lower the Dose of Prednisolone in Patients with Alopecia Areata

Ashwaq N. Al-Jaff**, Salim A. Humadi*, Saleh A. Wohaib**

Received 9-2-2003 Accepted 14-12-2003

ABSTRACT

Alopecia areata is a common disorder, hypothesized to be autoimmune in etiology. Cortisone taken orally may stimulate new hair growth. Prednisone (orally administered steroid) has proved effective for patients with alopecia areata, but its potential side effects include weight gain, metabolic abnormalities, acne and menstrual problems.

This clinical study was designed to assess the clinical significance of the nutrient antioxidants (vitamin A, vitamin E and vitamin C) in reducing the dose of corticosteroids (prednisolone), and as a consequence, their side effects in patient with alopecia. The results of this study reveal the potential clinical significance of the therapy for two months with these antioxidants in reducing the dose of prednisolone from 100mg to 10 mg administered each other day and improving the rate of hair growth by attenuating free radicals damaging effect on immune system, thereby decreasing the immune complex deposition. According to the results of this study, the use of nutrient antioxidants may have an important role in protecting the immune system, and decreasing the dose and side effects that result from the use of high dose of corticosteroids.

INTRODUCTION

Alopecia areata is a common, unpredictable, non-scarring form of hair loss (1,2,3,4). This disorder affects all age groups, with a higher prevalence in children and adolescents (4). The cause is unknown but it is associated with an alteration in the immunological system (5,6). Current treatment is not, at this point, directed at the etiology of alopecia areata but rather at the resulting inflammatory infiltrate and (presumably) the growth inhibitory factors produced by this response (5,6).

The use of nutrient antioxidants in alopecic patients revealed a significant decrease in basal and H2O2 induced MDA (biomarker of oxidative stress) level in RBC and plasma, increase glutathione level (major antioxidant) in both RBC and plasma, increase total protein and finally increase catalase activity. These effects suggest the important role of nutrient antioxidants in protecting the body( immune system from the oxidative damage produced by the disease) and may influence the severity of the disease(7).

Research has shown that the disease responds to a variety of immunomodulating treatments, that patients with alopecia areata may have a higher incidence of circulating antibodies against other body organs or tissues, and that family members have a higher incidence of autoimmune disease (5,5,6).

*Departments of clinical pharmacy, College of pharmacy, university of Baghdad, Baghdad- Iraq
**Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad – Iraq.
Research has shown that the disease responds to a variety of immunomodulating treatments, that patients with alopecia areata may have a higher incidence of circulating antibodies against other body organs or tissues, and that family members have a higher incidence of autoimmune disease (3, 5, 6).

Systemic steroids are reserved for use in rapidly progressive or extensive alopecia areata (8, 9, 10). Systemic steroids, particularly a short course (4-8 weeks) of tapering doses, are often used either alone or in combination with topical agents. A high dose up to 100 mg prednisolone daily has been recommended. In this setting acne and weight gain are commonly seen side effects (11). Prednisolone doses as low as 20 mg per day may be associated with septic necrosis of the hip or severe gastrointestinal bleeding (12, 13).

This study was designed to investigate the role of nutrient antioxidants (A, E, &C) in reducing the dose of prednisolone and as a consequence their side effects in patient with alopecia.

SUBJECTS and METHODS

1- Subjects
A-Study Group: comprised of total of 84 subjects, 30 normal controls (mean age 25.97± 8.09 years) and 54 cases with alopecia (mean age 25.20 ±7.05 years). Patients involved in this study were under a dermatologist supervision who determined the severity of the disease according to number of the patches they have, and according to progression of disease (2) they were non-smokers, non-alcoholics and free from apparent other diseases. The duration of disease ranged from (20 day- 18 years).

B-Patients: Fifty-four patients aged 10-40 years (26 females, 28 males) with alopecia (with no previous treatment) were included in this study. [Twenty seven of them received corticosteroids (100 mg prednisolone) each other day, and the other twenty seven receive (10 mg prednisolone) each other day]. Treatment schedules also included a combination of antioxidants [vitamin A (5000 IU/day), vitamin E (100 mg/day) and vitamin C (500 mg/day)] given to both groups. The treatment with nutrient antioxidants for alopecic patients included in this study continued for two months.

C-Samples: heparinized venous blood samples were collected from alopecic patients as well as from controls using plastic disposable syringes. Fresh blood sample were used for MDA and GSH measurements.

2-Methods
- Erythrocytes Malondialdehyde (MDA) Assay:
Measurements of erythrocyte and plasma MDA (which is a by product of lipid peroxidation), based on the reaction of thiobarbituric acid (TBA) forming TBA-MDA adduct, were carried out using the modified method of Stocks and Dormandy(16) as described by Gilbert et al(18). The results were expressed as nmole/g Hb and µmol/ L plasma based on the molar extinction coefficient of 105 × M1. CM1. × MDA is 1.56
- Glutathione Assay:
Erythrocytes and plasma GSH contents were determined according to the method of Godin et al.(16). Known amounts of GSH were assayed by the same method and used for calculation of GSH quantities in erythrocytes. The statistical significance of the difference in mean was tested by student t-test.

RESULTS
A- Basal plasma and erythrocyte MDA levels in both groups of patients were significantly higher than those in controls. Treatment with either 10 or 100 mg prednisolone plus antioxidants normalized MDA levels in both plasma (Table 1) and erythrocytes (Table 2) as early as 1 month after treatment.

Furthermore, total plasma GSH content was significantly higher than controls (Table 3), and treatment of patients with both doses of prednisolone plus antioxidants slightly increased GSH patients, but did not normalize these values. On the other hand, erythrocyte GSH content was significantly lower in patients compared to controls, and that treatment with both doses of prednisolone plus antioxidants did significantly elevate GSH content in patients after 1 month of treatment, and normalized these values after 2 month’s of treatment (Table 4).

B- Clinically there is lower incidence of prednisolone side effects( acne and weight gain) among those patients taking prednisolone dose 10 mg each other day than those taking 100 mg each other day ( Table 5 and 6 ).
Table (1): Effect of the addition of nutrient antioxidants (A, E, & C) to prednisolone therapy (10 & 100 mg) on plasma MDA levels in patients with alopecia areata.

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (μ mole /L)</th>
<th>Patients with alopecia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control N = 30</td>
<td>Pre-treatment N = 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Months after treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 N = 27</td>
</tr>
<tr>
<td>I- Antioxidants + 10 mg prednisolone</td>
<td>0.72±0.30</td>
<td>3.17±1.67 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.87± 0.45†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.66±0.27†</td>
</tr>
<tr>
<td>II- Antioxidants + 100 mg prednisolone</td>
<td>0.72±0.30</td>
<td>2.73±1.66 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.86± 0.46†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.67±0.26†</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
* Significantly different from control (p< 0.05).
† Significantly different from pretreatment values (p<0.05).
N Number of subjects

Table (2): Effect of the addition of nutrient antioxidants (A, E, & C) to prednisolone therapy (10 & 100 mg) on erythrocytes MDA levels in patients with alopecia areata.

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (n mole /g Hb)</th>
<th>Patients with alopecia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control N = 30</td>
<td>Pre-treatment N = 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Months after treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 N = 27</td>
</tr>
<tr>
<td>I- Antioxidants + 10 mg prednisolone</td>
<td>5.98±1.04</td>
<td>28.38±18.60 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.25±2.95†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.44±2.48†</td>
</tr>
<tr>
<td>II- Antioxidants + 100 mg prednisolone</td>
<td>5.98±1.04</td>
<td>28.40±18.84 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.27±3.09†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.46±2.60†</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
* Significantly different from control (p< 0.05).
† Significantly different from pretreatment values (p<0.05).
N Number of subjects
Table (3): Effect of the addition of nutrient antioxidants (A, E, & C) to prednisolone therapy (10 & 100 mg) on plasma glutathione levels in patients with alopecia areata.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (µ mol/L)</th>
<th>Control N=30</th>
<th>Patients with alopecia</th>
<th>Pre-treatment N=27</th>
<th>Months after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 N=27</td>
<td>2 N=27</td>
</tr>
<tr>
<td>I- Antioxidants + 10 mg prednisolone</td>
<td>0.90±0.20</td>
<td>1.21±0.35*</td>
<td>1.27±0.55*</td>
<td>1.54±0.77*</td>
<td></td>
</tr>
<tr>
<td>II- Antioxidants + 100 mg prednisolone</td>
<td>0.90±0.20</td>
<td>1.23±0.38*</td>
<td>1.27±0.57*</td>
<td>1.58±0.79*</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
* Significantly different from control (p<0.05).
N Number of subjects

Table (4): Effect of the addition of nutrient antioxidants (A, E, and C) to prednisolone therapy (10 and 100 mg) on erythrocytes glutathione levels in patients with alopecia areata.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (µ mole/gm Hb.)</th>
<th>Control N=30</th>
<th>Patients with alopecia</th>
<th>Pre-treatment N=27</th>
<th>Months after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 N=27</td>
<td>2 N=27</td>
</tr>
<tr>
<td>I- Antioxidants + 10 mg prednisolone</td>
<td>6.53±0.83</td>
<td>3.95±1.32*</td>
<td>5.43±1.39*</td>
<td>5.98±1.25†</td>
<td></td>
</tr>
<tr>
<td>II- Antioxidants + 100 mg prednisolone</td>
<td>6.53±0.83</td>
<td>4.06±1.39*</td>
<td>5.44 ±1.42*</td>
<td>5.99 ±1.29†</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
* Significantly different from control (p<0.01).
† Significantly different from pretreatment values (p<0.01).
N Number of subjects
Table (5): body weight of control and age matched alopecic patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (Kg)</th>
<th>Patients with alopecia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N=27</td>
</tr>
<tr>
<td>Control N=30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I- Antioxidants + 10 mg prednisolone</td>
<td>69.69 ±10.79</td>
<td>66.09±12.00</td>
</tr>
<tr>
<td>II- Antioxidants + 100 mg prednisolone</td>
<td>69.69 ±10.79</td>
<td>66.79±12.76</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.
N Number of subjects

Table (6): severity of acne appearance in control and age matched alopecic patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Presence of acne</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients with alopecia</td>
</tr>
<tr>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td></td>
<td>N=27</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Control N=30</td>
<td></td>
</tr>
<tr>
<td>I- Antioxidants + 10 mg prednisolone</td>
<td>negative</td>
</tr>
<tr>
<td>II- Antioxidants + 100 mg prednisolone</td>
<td>negative</td>
</tr>
</tbody>
</table>

Severity of the presence of acne determined by dermalogists.
N Number of subjects

**DISCUSSION**

Corticosteroids are part of the treatment of many disorders in which inflammation is thought to be caused by excessive or inappropriate activity of the immune system like in Alopecia areata (17, 18, 19, 20, 21). Given in high doses, corticosteroid drugs reduce inflammation by blocking the action of prostaglandins responsible for triggering the inflammatory response (16). They also temporarily depress the immune system by reducing the activity of certain types of white blood cells. The extent of hair loss and the age of the patient are used to select an appropriate treatment for patients with alopecia areata (3). For those with more than fifty percent scalp hair loss one may consider the use of systemic corticosteroids but the concern about long-term use and side effects of systemic corticosteroids must be taken into consideration.

The present study revealed the presence of endogenous oxidative stress in both groups of patients, as manifested by the increased MDA.
levels and decreased GSH contents in erythrocytes. This oxidative stress may result from phagocytes derived free radicals and the associated lipid peroxidation\(^2\). Data of the present study also indicated that, despite the difference in oral prednisolone dose (10 vs. 100 mg) between the two groups, addition of nutrient antioxidants to prednisolone therapy resulted in comparable and significant decrease in MDA levels and correction of GSH content in blood, as well as similar improvement in the rate of hair growth with less side effect (acne, weight gain and gastrointestinal disturbances) regardless the dose of prednisolone. Previous study in our lab showed that, without antioxidant therapy, the effect of 100 mg prednisolone was more effective than lower doses of prednisolone in improving hair growth in alopecia areata patients\(^7\).

Therefore, the addition of nutrient antioxidants to corticosteroids attenuated the negative effects of oxidative stress on immune system and decreased the need for high dose of corticosteroid; thereby decreased the unwanted side effects associated with the prolonged use of high doses.

**Free radicals → Affect immune system → Phagocytosis → Increase free radical production**

Antioxidants

Antioxidants

**REFERENCES:**

Synthesis and Characterization of 2(2-Tetrahydropyrylthio) Methyl Cyclopropylamine

Zuhair A. Muhi-Eldeen*, Samira F. Hassan**

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ABSTRACT
2(2-Tetrahydropyrylthio) methyl cyclopropyl amines were synthesized from allylmercaptan through several steps. The structures of the intermediates and the final products were confirmed through IR, NMR and elemental analysis, these compounds may be of value in the treatment of diseases where free radicals are implicated in their pathogenesis, since the thio and the amino groups of the synthesized compounds may act as free radical scavengers.

INTRODUCTION
Free radicals have been implicated in many diseases, among these are atherosclerosis, rheumatoid arthritis, cataracts, neoplastic disease, diabetic retinopathy, Parkinson's, Alzheimer inflammatory diseases of gastro-intestinal tract and aging. Free radicals are defined as atoms or molecules that contain one or more unpaired electrons and are species that cause metabolic disturbances and cell injury by interacting with macromolecules and other cellular constituents such as proteins, lipids, carbohydrates and DNA resulting in a variety of biological consequences, including cellular and tissue damage, mutation carcinogenesis and cell death. The observation that 2-mercaptoethane, 2-mercaptoethylamine, disulfide, Thioethers and sulfoxides were capable in protecting animals against free radicals generated as a result of ionizing radiation promoted our interest to synthesize-2(2-tetrahydropyrylthio) methylcyclopropylamine, the thioether and the amino groups in L or the corresponding "sulfhydryl and amino groups in their expected major metabolites may act cooperatively as free radical scavengers". Therefore these compounds may be utilized selectively to treat one or more of the previously mentioned diseases.

SYNTHETIC MATERIAL
Allylmercaptan, p-toluensulfonic acid, dihydropyran ethldiazocetate, were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Analytical Equipment
Melting points were determined by using a calibrated Thomas Hoover melting point apparatus. IR spectra were recorded using a Unicam SP-300 spectrophotometer. NMR spectra were obtained using a Variant FT80A spectrometer. Chemical shifts are reported as part per million downfield from tetramethylsilane as internal standard for HNMR spectra. Elemental microanalysis were performed by H. Malissa and G. Reuter, FRG.

2- Allylthiotetrahydropyran (3).
- Allylmercaptan (3.74g, 1 mole) and 200 mg of p-toluene sulfonic acid were placed in a 500ml RB- flask fitted with a reflux condenser and magnetic stirrer. Dihydropyran (84g, 1 mole) was added dropwise. The reaction mixture was heated on a steam bath for 10 minutes. After heating for 5-10 minutes, a vigorous exothermic reaction started and continued during the addition of dihydropyran. After 1 1/2 hours, refluxing was stopped and potassium carbonate (1.0 g) was added. The mixture was stirred at room temperature for 1 hour, filtered and fractionally distilled yielding 80.2 g. (50%) of 2-allylamylthiotetrahydropyran (3) b.p. 42-44 (0.1 mm)

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Infrared (neat, cm⁻¹) showed bands at 3080 (= CH₂, stretch; 2940, 2860, 2850, (CH₂, stretch); 1635 (C = C, stretch); 1180, 1080, 1040, 1015 (tetrahydropyranyl group) and 940 (S-CH₂) Nmr (d- chloroform, δ) 1.28 - 2.18 (multiplet, 6 H, (CH₂)₃; 2.20 (multiplet, 2H, S-CH₂); 4.09 and 3.5 (multipletEt, 2H, OCH₂). The vinyl protons appear as multiplets overlapping with (O-CH₂) at 5.20 (multiplet, 3H, C=CH₂, O-CH₂) and 5.82 (multiplet, 1H, C=CH).

Anal. Calculated: Found for C₈H₁₄O₇S: C, 60.75; H, 8.86; S, 20.25. Found: C, 60.63; H, 8.85; S, 20.47.

2-Tetrahydroxypyranyltio)methyl-1-carboethoxycyclopropane (4)

5.5 g (22.5%), of 4 was obtained as a colorless liquid, b.p. 120-122°C at (0.2 mm), gas liquid partition chromatography on 3.8% silicon gum rubber (UC-W98) on chromosorb W (80-100 mesh), 4ft, 0.25 in glass column with column temperature 190°C, injection part temperature 320°C, detector temperature 280°C, inlet pressure of 40 psi and carrier gas (N2) flow rate of (60 ml/min) showed two peaks at 3.2 minutes (87%) and 4.0 minutes (13%) cis-4. The mixture had an infrared spectrum (neat cm⁻¹) that showed bands at 2900, 2860 (CH₂, stretch); 1720 (C=O, stretch); 1105, 1080, 1040 and 1015 (tetrahydropyranyl group and cyclopropane absorption). NMR (d-chloroform, δ) 0.7-1.2 (multiplet, 2H, CH₂ of cyclopropane); 1.34 (triplet, 3H, CH₃); 1.4-2.15 (multiplet 7H(CH₃); 1H of cyclopropane α to CH₃-S); 2.58 (doublet, 2H, S-CH₂-J, CH₂-S = 6.5 Hz) 3.3 (multiplet, 1H, CH-cyclopropane α to COOEt); 3.56 (multiplet, 1H, β-CHO of tetrahydropyranyl group); 3.9 (multiplet, 1H, α-CHO of tetrahydroxypyranyl group), 4.14 (quartet, 2H, CH₂ of the ester group), 5.0 (multiplet, 1H, O-CH₂-S).


2-(2-Tetrahydroxypyranyltio)methyl-1-carboethoxycyclopropane (4)

A solution of (4.88 g, 2.0x10⁻³ mole) of 2-(2'-tetrahydroxypyranyltio)methyl-1-carboethoxycyclopropane 4 and 20 ml of 85% hydrazine hydrate was refluxed for 24 hours. The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch). The mixture was cooled and held at 0°C for 24 hours; no precipitate formed. The excess of hydrazine hydrate was removed under reduced pressure affording a semi-solid that failed to crystallize under various conditions. The unreacted ester 4 was removed by dissolving the crude hydrazide in chloroform. The hydrazide 6 was precipitated with anhydrous ether. A white solid was obtained in Et₂O; this turned to a semi-solid upon removal of ether. The hydrazide 6 had an infrared spectrum (neat cm⁻¹) that showed bands at 3300 (NH); 3080 (C=CH₂, stretch); 1660 (CONH, stretch); 1750 (C=O, stretch).
NH₂, CH₂-cyclopropane) ; 2.6 (multiplet, 1H, S-CH₂) 1.1-1.2(multiplet,7H,(CH₃) and 1H, CH of cyclopropane α- to CH₂-S); 0.8- 1.0 (multiplet,2H, CH of cyclopropane ). This semi-solid hydrazide was used without further purification.

2(2Tetrahydropyranylthio)methylclopropylamine

A solution of (4.64g,2.0x10⁻² mole) of 2-(2-tetrahydropyranylthio) methylclopropylcarboxyhydrazide 6 in 100ml chloroform was chilled to -5°C (ice-salt bath). With rapid stirring, 1.2g(2.0x10⁻² mole) of sodium nitrite in 5ml of water, followed by 10ml of 10% HCl, were added. The reaction mixture was allowed to stand 15-20 minutos; the chloroform layer was separated and the aqueous layer was extracted once with 20ml of chloroform. The combined chloroform fractions were dried (Na₂SO₄) and filtered. To this solution 50ml of dry toluene was added. The chloroform was removed under reduced pressure and N₂ evolution continued vigorously while heating the remaining toluene solution under reduced pressure on steam bath. After N₂ evolution ceased a dark brown solid material precipitated (infrared, KBr, 2280 cm⁻¹ (N=C=O, 8)). Fifteen ml of 25% methanolic KOH was added to the toluene containing the solid isocyanate and the mixture was refluxed at 125°C for 18 hours. After heating, the reddish brown mixture was extracted with toluene, dried (Na₂SO₄) and filtered. The toluene was removed under reduced pressure and the residue was distilled affording 0.5g(26.7%) of cis- and trans-amine 1b, p. b. 98 -100°C at (0.3mm), Gass liquid partition chromatography (g LPC) analysis on 3.8% silicon gum rubber (UC- W-98) on chromosorb - W (80-100 mesh), 4ftx0.25 in glass column temperature 175°C, injection part temperature 300°C and detector temperature 285°C inlet pressure 40 psi and carried gas (N₂) flow rate of (60ml /min) showed two peaks at 1.28 minutes (85%) trans-1 and 2.48 minutes (15%) cis-1.

The amine mixture had an infrared spectrum (neat, cm⁻¹) that showed bands at 3600-3300(broad, NH₂ Stretch); 3000, 29 30 and 2860 (CH₂-stretch) 1625-1590(NH, bending); 1105,1080,1045 and 1015 (tetrahydropyranyl and cyclopropyl ring). NMR (d-chloroform, δ 4.97) : (multiplet, 1H, O-CH-S); 4.0 nd 3.5(multiplet,2H,O-CH₂); 2.6 (multiplet,2H, S-CH₂) 2.3 (multiplet, 1H, CH, cyclopropane α- to NH₂); 1.1 - 2.2 (multiplet,7H,(CH₃); 1H, CH, cyclopropane α- to CH₂-S), 0.7 - 1.1 (multiplet, 2H, CH₂,cyclopropane).

Analysis: Calculated: Found For C₇H₇O₃N₂S: C, 57.75; H, 9.09; N, 7.48; S, 17.11. Found : C, 57.19; H, 8.84; N,7.03; S,18.44.

RESULTS and DISCUSSION

The new compound 1 was prepared as depicted in scheme 1 Allymercaptan 2 which serves as starting material was readily converted in 80% yield to 2-allylthiotetrahydropyran 3 through reaction with 2,3-dihydropyran in the presence of p-toluenesulfonic acid. The IR and NMR spectra were consistent with the assigned structure. Treatment of 3 with ethylidiazocetate afforded a mixture of trans- and cis- 2-(2-tetrahydropyranylthio) methyl-1-carboethoxycyclopropane 4 and a sulfonium ylide rearrangement product namelyethyl-α-allyl-α (2-tetrahydropyranylthio) acetate 5 in 81.4% and 16.8% yield at 150-155°C respectively. Ethylidiazocetate reacts with allymercaptan to generate trans- and cis-cyclopropane derivative 4 through carbene addition to the double bond and with thioether group to form sulfonium ylide. Such sulfonium ylide is known to undergo Steven's rearrangement, which depends on the structure of the ylide, may either involve an antrafacial 1,3-sigmatropic rearrangement or suprafacial 1,5-sigmatropic rearrangement. In our case both 1,3- and 1,5- rearrangements afforded the same compound 5. The mixture of 4 could not be separated by physical methods and was used as a mixture in the next step. Reaction 4 with 85% hydrazine hydrate afforded the hydrazide 6 as a gummy solid in almost quantitative yield; Attempts to crystallize this gummy hydrazide were unsuccessful. The purity of the compound was determined by gas liquid partition chromatography (g LPC); the non-crystalline hydrazide was then subjected to Curtius rearrangement. The rearrangement proceeded through the azide 7 and the isocyanate 8. The intermediate azide 7 was detected by its IR spectrum (CON₃, 2150 cm⁻¹). The isocyanate 8 could be isolated as a brown solid which showed an IR absorption band at 2280 cm⁻¹ (N=C=O). The isocyanate 8 was refluxed with 25% methanolic KOH to generate the desired trans- and cis- cyclopropylamine 1 as a mixture in a ratio of 85:15 respectively. The IR and NMR spectra were consistent with the assigned structure as discussed in the experimental part.
REFERENCES


The Effects of Melatonin On The Oxidative Stress, Protein Glycation, Microalbuminuria and Lipid Profile In Type II Diabetes Mellitus

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ABSTRACT

Previous studies indicated that supplementation with antioxidants has a protective effects against oxidative stress—induced damage in type 2 diabetes. In this study we evaluated the antioxidant effects of melatonin on the oxidative stress parameters and microalbuminuria in type 2 DM patients. 30 patients with type 2 DM were treated with 3mg/day melatonin for 90 days. Erythrocytes and plasma MDA and glutathione, fasting plasma glucose, %HbA1C, microalbuminuria, total plasma protein and lipid profile were measured each 30 days and compared with those obtained from 20 healthy controls. A decrease in MDA levels associated with the elevation in GSH levels were observed, compared with the pretreatment levels. Fasting plasma glucose, glycated hemoglobin and microalbuminuria were significantly decreased, associated with an improvement in the total cholesterol, HDL–C and LDL–C levels, with respect to the pretreatment values. In conclusion , treatment of type 2 DM patients with melatonin may have protective effects against the oxidative stress—induced damage during the course of type 2 DM.

Key words: Diabetes Mellitus, Oxidative Stress, Melatonin, Microalbuminuria.
However, despite the widespread use of melatonin, there is minimal information on its use as a pharmacological approach in the treatment of DM and its complications. There is also minimal information on the toxicology of high pharmacological doses (14). Based on LD50 values in animals, it appears that melatonin has an extremely low acute toxicity (15).

This study determined whether treatment with melatonin altered the clinical and biochemical abnormalities associated with DM in patients with type II diabetes mellitus.

**PATIENTS and METHODS**

This study was carried out on 30 patients who have type II DM for at least 5 years, with age range (40–80 years) at the National Diabetes Center, Al–Mustansiria University. All the selected patients were previously maintained on oral hypoglycemic drugs and diet restriction, but with poor glycemic control. They are treated with 3 mg/day of melatonin in a capsule dosage form, prepared specially for this purpose, given orally at bed time for three months.

Twenty healthy subjects, with the same age ranges as that of patients were selected and served as controls for comparison.

Fasting blood samples were collected from all subjects by vein puncture, before starting treatment with melatonin (as base line samples), and then after each 30 days of treatment to follow the changes in the studied parameters.

All blood samples were collected in heparinized tubes. Erythrocytes were separated by centrifugation at 3000 r.p.m. for 10 min. at 4°C, after the removal of the puffy coat, the erythrocytes were washed twice with ice-cooled saline containing 2.5 mM sodium azide to inhibit catalase activity (20). Urine samples were collected from all subjects at the early morning before starting treatment and every 30 days for the evaluation of microalbuminuria (17).

Erythrocytes and plasma malondialdehyde (MDA) levels were analyzed according to the method of Stocks and Dormandy (1971) (17). Erythrocytes and plasma glutathione levels were measured according to the method of Godin et al. (1988) (18). Plasma glucose levels were evaluated using a ready made kit (LABKIT, Spain) according to the method of Barhan and Trindoer (1972) (19), and glycated hemoglobin (HbA1c) level was determined according to the method of Sushil (2000) (20). Total plasma protein concentrations were determined according to the Reinhold (1953) method (21), while hemoglobin (Hb) levels were estimated according to the method of Drapkin and Austin (1935) (22).

Plasma lipid profile was evaluated through the measurement of total plasma cholesterol according to the method of Richmond (1974) (23), and triglycerides levels according to the method of Fossati and Principe (1982) (24), while Burstein et al. (1970) (25) method was utilized for the measurement of high density lipoprotein–cholesterol (HDL–c) levels from which the plasma concentrations of the low density lipoprotein–cholesterol (LDL–c) levels were calculated indirectly using Burstein and Ashwood formula (1999) (26). Statistical analysis of data was done by two–way comparison of mean values, utilizing Student’s t–test, P–values less than 0.05 was considered significant.

**RESULTS**

Combination of the routinely used measures for glycemic control with 3 mg/day melatonin, produced significant reduction in MDA levels in plasma (after 60 days), and erythrocytes (after 30 days) Table (1). The level of reduction in MDA reached values which are comparable, or even lower than that found in corresponding controls after 90 days of treatment.

<table>
<thead>
<tr>
<th>Table (1): Effects of treatment with 3mg/day melatonin on plasma and erythrocytes MDA levels in type 2 diabetic patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment period</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Zero time</td>
</tr>
<tr>
<td>30 days</td>
</tr>
<tr>
<td>60 days</td>
</tr>
<tr>
<td>90 days</td>
</tr>
</tbody>
</table>

Results represent mean ± standard error. n = number of subjects.
* Significantly different with respect to control ( P < 0.05 ).
** Significantly different with respect to zero time ( P < 0.05 ).
Table (2) showed that the oxidant stress-induced depletion of GSH in the plasma was improved after 30 days of treatment with 3 mg/day of melatonin (58%), and continuous treatment led to further elevation in plasma GSH levels, which nearly matched their values in normal controls after 90 days of treatment.

Table (2): Effects of treatment with 3mg/day melatonin on plasma and erythrocytes glutathione (GSH) levels in type 2 diabetic patients

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>n</th>
<th>Plasma GSH μmol/L</th>
<th>Erythrocytes GSH μmol/g Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>0.36 ± 0.07</td>
<td>8.29 ± 0.1</td>
</tr>
<tr>
<td>Zero time</td>
<td>30</td>
<td>0.13 ± 0.01*</td>
<td>6.26 ± 0.43*</td>
</tr>
<tr>
<td>30 days</td>
<td>30</td>
<td>0.21 ± 0.01**</td>
<td>6.79 ± 0.57</td>
</tr>
<tr>
<td>60 days</td>
<td>30</td>
<td>0.27 ± 0.02**</td>
<td>6.8 ± 0.42</td>
</tr>
<tr>
<td>90 days</td>
<td>30</td>
<td>0.29 ± 0.02**</td>
<td>7.0 ± 0.51</td>
</tr>
</tbody>
</table>

Results represent mean ± standard error.

n = number of subjects.

* Significantly different with respect to control (P < 0.05).

** Significantly different with respect to zero time (P < 0.05).

However, erythrocytes GSH levels which are severely depleted due to DM–induced oxidant stress, showed a non–significant increase (P > 0.05), even after 90 days of treatment with melatonin.

As a result of treatment with 3 mg/day melatonin for 90 days, fasting plasma glucose (FPG) and glycated Hemoglobin (Hb_A1C) levels were significantly reduced (26% and 11% respectively) compared with pretreatment levels (Table 3).

Table (3): Effects of treatment with 3mg/day melatonin on fasting blood glucose (FPG) and Glycated hemoglobin (Hb_A1C) levels in type 2 diabetic patients.

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>n</th>
<th>FPG mg/dl</th>
<th>Hb_A1C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>94 ± 4.6</td>
<td>5.4 ± 0.28</td>
</tr>
<tr>
<td>Zero time</td>
<td>30</td>
<td>190 ± 22*</td>
<td>9.46 ± 0.6*</td>
</tr>
<tr>
<td>30 days</td>
<td>30</td>
<td>153 ± 15</td>
<td>-</td>
</tr>
<tr>
<td>60 days</td>
<td>30</td>
<td>173 ± 17</td>
<td>8.7 ± 0.59</td>
</tr>
<tr>
<td>90 days</td>
<td>30</td>
<td>135 ± 11**</td>
<td>7.2 ± 0.4**</td>
</tr>
</tbody>
</table>

Results represent mean ± standard error.

n = number of subjects.

* Significantly different with respect to control (P < 0.05).

** Significantly different with respect to zero time (P < 0.05).

As indicated in Table (4), microalbuminuria and total plasma protein levels were severely affected by DM–induced oxidant stress, where 52% increase and 16% decrease in both parameters were observed (Table 4) respectively, and were significantly different compared to controls.

Table (4): Effects of treatment with 3mg/day melatonin on the microalbuminuria and total plasma protein levels in type 2 diabetic patients.

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>n</th>
<th>Microalbuminuria mg/L</th>
<th>Total plasma protein g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>169 ± 4.1</td>
<td>8.55 ± 0.34</td>
</tr>
<tr>
<td>Zero time</td>
<td>30</td>
<td>258 ± 38*</td>
<td>7.23 ± 0.12*</td>
</tr>
<tr>
<td>30 days</td>
<td>30</td>
<td>169 ± 43</td>
<td>7.47 ± 0.09</td>
</tr>
<tr>
<td>60 days</td>
<td>30</td>
<td>157 ± 41**</td>
<td>7.35 ± 0.08</td>
</tr>
<tr>
<td>90 days</td>
<td>30</td>
<td>140 ± 30**</td>
<td>8.9 ± 0.1**</td>
</tr>
</tbody>
</table>

Results represent mean ± standard error.

n = number of subjects.

* Significantly different with respect to control (P < 0.05).

** Significantly different with respect to zero time (P < 0.05).
Melatonin treatment resulted in significant reduction in microalbuminurea (39%) after 60 days, with further decrease after 90 days (42%). However, total plasma protein levels started to show significant elevation (P < 0.05) after 90 days of treatment only (Table 4).

Table (5) clearly demonstrated that, the abnormal lipid profile in DM state, respond very well to melatonin treatment, where the elevated total cholesterol values started to decrease significantly (20%, P < 0.05) after 60 days, reaching a level which was lower than that observed in controls after 90 days. Triglyceride levels showed (22%) decrease after 90 days of treatment, but this was still non–significant compared with pre–treatment values.

Table (5): Effects of treatment with 3mg/day melatonin on the lipid profile in the plasma of type 2 diabetic patients

<table>
<thead>
<tr>
<th>Treatment period</th>
<th>n</th>
<th>TC mg/dl</th>
<th>TG mg/dl</th>
<th>HDL-c mg/dl</th>
<th>LDL-c mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>184 ± 5.8</td>
<td>114 ± 18.1</td>
<td>37 ± 0.95</td>
<td>125 ± 5.8</td>
</tr>
<tr>
<td>Zero time</td>
<td>30</td>
<td>211 ± 16.7</td>
<td>211 ± 33.7*</td>
<td>33 ± 0.83*</td>
<td>132 ± 14.2</td>
</tr>
<tr>
<td>30 days</td>
<td>30</td>
<td>170 ± 15.1</td>
<td>201 ± 29.9</td>
<td>38 ± 1.9**</td>
<td>100 ± 11.1</td>
</tr>
<tr>
<td>60 days</td>
<td>30</td>
<td>168 ± 9.4**</td>
<td>183 ± 22.8</td>
<td>42 ± 3.5**</td>
<td>84 ± 8.2**</td>
</tr>
<tr>
<td>90 days</td>
<td>30</td>
<td>147 ± 11**</td>
<td>164 ± 18.9</td>
<td>43 ± 3.3**</td>
<td>71 ± 9.2**</td>
</tr>
</tbody>
</table>

Results represent mean ± standard error.

n = number of subjects.

* Significantly different with respect to control ( P < 0.05 ).

** Significantly different with respect to zero time ( P < 0.05 ).

HDL–c levels started to increase significantly after 30 days of treatment with melatonin (15%, P < 0.05). Maximum increase in HDL–c value was observed after 90 days of treatment (30%), which is higher than that observed for controls (Table 5). However, LDL–c levels started to decrease as a response for melatonin treatment only after 60 days of treatment (36%, P < 0.05). After 90 days, 46% decreases in LDL–c values were observed, which is highly significant compared with pre–treatment values, and even lowered than those observed in controls.

DISCUSSION

Melatonin has been suggested to have potent antioxidant properties that may prevent the development of cancer, atherosclerosis, and other consequences of aging, however, these hypothetical effects are unproven (27). Thus conclusive studies regarding the relevance of antioxidant properties of melatonin in the prevention of diseases, like diabetes mellitus, and their complications are not widely available. In this study, we evaluated the possible antioxidant activity of melatonin in ameliorating the oxidant stress state during DM, and the results shown in table (1) very well indicated that this low dose of melatonin resulted in significant reduction in MDA production in plasma and erythrocytes of DM patients. This result clearly indicated the promising antioxidant activity, especially when correlated with the observed elevation of the soluble antioxidant, the glutathione (table 2). Higher doses may be required to achieve more improvement in the antioxidant profile, since animal studies showed that, for melatonin to show a potent antioxidant activity in vivo, very high daily doses may be required (10 mg/kg/day)(28). Researchers have discovered melatonin to have the most powerful antioxidant properties, it scavenges the most damaging free radical, the hydroxyl radical, five times better than GSH, and is twice as effective in deactivating the peroxyl radical as vitamin E (29).

Montilla et al. (1998) demonstrated that melatonin show a marked protective effect against oxidant stress resulted from hyperglycemia and protein glycosylation in experimental animals, two pathogenic cornerstones indicative of diabetic complications (29).

The results presented in table (3), indicated a significant reduction in FPG and HbA1c levels after 90 days of treatment with melatonin. The presented data and the preliminary findings of others (30) suggest that melatonin is protective against DM–induced damage, even at physiologic levels. Even if only pharmacologically relevant, the findings have important implications in that melatonin has no known toxicity and readily absorbed when administered by oral rout.
Antioxidants now a days are found in many clinical trials, to play a role in the amelioration of the microvascular changes during diabetic nephropathy, and this was clearly demonstrated by Nakamura et al. (1999) who observed the beneficial effects of Vit. E on microalbuminuria in DM nephropathy. Table (4) clearly demonstrated the effect of a 3 mg/day melatonin in the reduction of microalbuminuria in 60 days, and this consequently reflected on the improvement of total plasma protein after 90 days, and these results seems to be compatible with those observed by Ha et al. (1999), where treatment with melatonin decrease the incidence of early glomerulopathy in diabetic rats.

The ability of melatonin to inhibit the impairment of lipid profile in DM patients was studied, and the results presented in table (5) showed that treatment with 3 mg/day melatonin improves the lipid profile through the reduction of TC and LDL-c levels, associated with significant elevation in HDL-c levels. It is postulated that inhibition of LDL-c oxidation by antioxidants might protect against the development of atherosclerosis, and it is noted that, in both human and animal studies, resistance of LDL-c to oxidation has been associated with decreased vascular complications, and protection by antioxidants decreases susceptibility to vascular disturbances due to other diseases like DM.

Recent studies suggested that HDL-c has an antioxidant effect on LDL-c, thus Klimov et al. (1993) reported that HDL-c was protective against LDL-c oxidation, and this effect was concentration dependent. Therefore, the use of melatonin alone or with other antioxidants, may contributed to the protection of LDL-c oxidation and restore the endogenously present HDL-c molecules to play the protective role effectively.

In conclusion, melatonin through reducing remarkably the degree of lipid peroxidation, hyperglycemia, protein glycation, might give a hope to a promising perspective of this product in the treatment of diabetic complications.

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Effect of Metformin and Antioxidant Agents on Hirsutism in Women with Polycystic Ovary Syndrome
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ABSTRACT
Forty six Iraqi women with PCOS were involved in this study. They were treated with metformin alone and with antioxidant agents (vitamin E or C). It was found that all patients who treated with metformin or with combination of metformin with antioxidant agents showed significant decrease in hirsutism score. The treatment of metformin with antioxidant agents is of great benefit in treatment of hirsutism in PCOS due to that there was no worsening effect after treatment. This may indicate that antioxidant agents may participate in alleviation of hirsutism so it can be said that oxidative stress may play an important role in developing of hirsutism in PCOS.

INTRODUCTION
Hirsutism is the presence of excess hair growth in women and affects 5% - 8% of the total female population of fertile age (1). The hirsutism in polycystic ovary syndrome (PCOS) is mainly caused by ovarian androgen overproduction and in idiopathic hirsutism there is peripheral hypersensitivity to normal androgen circulating levels (2). 5α-reductase type 2 has been studied for the differentiation of external genitalia and prostate in males and is essential for hair growth in both sexes (3). A prerequisite for the cellular action of androgen on the pilosebaceous unit is the conversion of testosterone to dihydrotestosterone through the enzymatic action of 5α-reductase (4). However the women with PCOS may amplify the manifestation of hirsutism by the available of insulin growth factor -1 due to induction of 5α-reductase activity which is mediated by insulin growth factor -1 (5).

Antiandrogens are substances that prevent androgens from expressing their activity at target sites. The inhibiting effects of antiandrogen are different from those compounds that decrease release or inhibit biosynthesis of hypothalamic and anterior pituitary hormones (6). Oral contraceptive treatment of hirsutism is most effective in women with ovarian hyperandrogenism especially with PCOS. The attempt to alleviate hirsutism caused by androgen excess with oral contraceptive represents temporary measure. The preferred treatment is to target the pathogenic site of hyperandrogenism or the site of action. Metformin treatment of women with PCOS results in a decline of insulin as well as total and bioavailable testosterone leading to significant improvement of clinical manifestations of hyperandrogenism (7). Therefore the use of metformin in PCOS may afford an alternative way in management of hirsutism.
MATERIALS and METHODS
Forty–six Iraqi PCOS women with mean age (28 ±4.5 years) and eleven control women with comparable age were enrolled in this study. The patients were attending the outpatients clinic at Al-Elwea Hospital for Obstetric & Gynecology (Baghdad) and they had a mean hirsutism score (20.8±3.6) according to the modified Ferriman – Gallwey scoring system (8).

The PCOS women had menstrual irregularities (oligomenorrhea), chronic anovulation, obesity; elevated serum androgen levels, a serum LH/FSH ≥ 2 and typical arrested follicles were shown by ultrasound study. All patients had normal fasting glucose levels and normal markers of thyroid, and kidney function. None of them had taking any drug for at least 6 months before enrollment. The patients were classified according to the type of treatment:

**Group I**: 16 patients were treated with metformin (500 mg t.i.d).

**Group II**: 14 patients treated with combination of metformin (500 mg t.i.d) and vitamin C (250 mg t.i.d).

**Group III**: 16 patients were treated with combination of metformin (500 mg t.i.d) and (vitamin E 200 mg t.i.d).

The patients in all these groups were maintained on the treatment for a period of three months.

Clinical Study:-
A modified Ferriman – Gallwey System was used to clinically grade body hair growth (8). In particular, the degree of hirsutism was rated on a scale from 0 to 4 on 8 body regions. The hirsutism score was obtained by totaling the score for each body region and was determined before the study and again after each month for a period of 3 months of treatment. The score evaluation was performed by a single physician who was unaware of the treatment.

Biochemical Analysis:-
Blood samples for serum testosterone were collected before the treatment and after intervals of 1 month of treatment for 3 successive months. The serum levels of testosterone were measured by TESTO-CTZ which is radioimmunoassay Kit (CIS bio international – ORIS Group – France).

Statistical Analysis:-
Values are expressed as means ± SD. Student’s paired test was used for comparison of the parameters before and after treatment. A p value < 0.05 was considered statistically significant.

RESULTS
The age of the patients in group 1 was (29.2 ±2.9 years ), in group 2 the mean age was ( 27±3.9 years ) and in the group 3 the mean age was ( 30.1±3.6 years ). The basal values of total serum testosterone of each group are shown in table 1.

<table>
<thead>
<tr>
<th>Control levels</th>
<th>Base line levels of patients</th>
<th>After 1 month of treatment</th>
<th>After 2 months of treatment</th>
<th>After 3 months of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 ± 0.37 (n=11)</td>
<td>4.1 ± 1.3** (n=16)</td>
<td>2.4 ± 1.5**</td>
<td>2.4 ± 1.02**</td>
<td>1.5 ± 0.7**</td>
</tr>
<tr>
<td>1.1 ± 0.37 (n=11)</td>
<td>4.5 ± 1.7** (n=14)</td>
<td>3.1 ± 1.2**</td>
<td>2.5 ± 0.92**</td>
<td>2.3 ± 1.2**</td>
</tr>
<tr>
<td>1.1 ± 0.37 (n=11)</td>
<td>5.2 ± 2.2** (n=16)</td>
<td>3.31 ± 1.2**</td>
<td>3 ± 1.5**</td>
<td>2 ± 0.7**</td>
</tr>
</tbody>
</table>

**P<0.05
n=number of the subjects
Clinical and Hormonal Effects:
Clinical results are summarized in table 2 and figures 1, 2 and 3.

Table - 2 -
Effect of metformin and antioxidant agents on hirsutism score in PCOS patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before treatment</th>
<th>After 1 month</th>
<th>After 2 months</th>
<th>After 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20.5±5.3</td>
<td>19.3±5.4*</td>
<td>19.7±5.5*</td>
<td>16.6±7.7*</td>
</tr>
<tr>
<td>II</td>
<td>20.8±20</td>
<td>19±1.75*</td>
<td>17.5±3.8*</td>
<td>16±4.8*</td>
</tr>
<tr>
<td>III</td>
<td>21.2±2.2</td>
<td>19.1±5.4**</td>
<td>16.1±5.4**</td>
<td>14.2±6.3**</td>
</tr>
</tbody>
</table>

*P<0.05  
**P<0.005

Fig.1: Hirsutism score after treatment with metformin

Fig.2: Hirsutism score after treatment with Combination of metformin and vitamin C

Fig.3: Hirsutism score after treatment with combination of metformin and vitamin E

In all groups metformin and antioxidant agents decreased the hirsutism score significantly after 1, 2 and 3 months of treatment. The levels of serum testosterone in all groups were higher than normal levels and the serum testosterone decreased significantly in all groups after 1, 2 and 3 months of treatment (table - 1).

Evaluation of Clinical Outcome
The evaluation of the clinical outcome at the end of the treatment is summarized in table -3.

Table - 3 -
Rating of clinical outcome at the end of treatment

<table>
<thead>
<tr>
<th>Rating</th>
<th>No. (group 1)</th>
<th>No. (group 2)</th>
<th>No. (group 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excellent</td>
<td>1(6.25%)</td>
<td>2(14.2%)</td>
<td>6(37.5%)</td>
</tr>
<tr>
<td>Good effect</td>
<td>4(25%)</td>
<td>4(28.5%)</td>
<td>4(25%)</td>
</tr>
<tr>
<td>No effect</td>
<td>9(56.2%)</td>
<td>8(57.1%)</td>
<td>6(37.5%)</td>
</tr>
<tr>
<td>Bad effect</td>
<td>2(12.5%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The patients treated with metformin showed 12.5% bad (worsening) effects while the patients treated with combination of metformin and antioxidant agents show no worsening effects. The patients treated with metformin and vitamin E reported 37.5% of an excellent effect.

DISCUSSION
The common underlying causes of hirsutism are PCOS and idiopathic hirsutism. The goal of treatment is to interrupt the steps leading to the increased androgen expression of the pilosebaceous unit. Several strategies are available. Mechanical hair removal can improve hirsutism, but it is a temporary measure. In most cases hirsutism results from a combination of mildly increased androgen production and increased skin sensitivity to androgen. The polycystic
Ovary syndrome is characterized clinically by a history of chronic anovulation in combination with some evidence of androgen excess, such as histurism and acne. Many drugs with antiandrogenic properties, such as cyproterone, spironolactone, and flutamide, have been used to treat histurism in PCOS, but the efficacy of these drugs has been shown to be only partial. The hyperinsulinemia in PCOS was recognized as the cardinal manifestation of insulin resistance. It was hypothesized that in PCOS, insulin may directly stimulate ovarian cytochrome P450c17α, resulting in an increase production of androstenedione which is then may converted to testosterone by the enzyme 17β-reductase. However the use of metformin in this study may resolve the main challenge of PCOS which is insulin resistance and this may reduce the action of insulin on the ovary and consequently reduce the overproduction of testosterone (table 1). This result is in agreement with that of Kolodziejczyk et al. who found that metformin treatment with PCOS results in improvement of clinical manifestation of hyperandrogenism. Recently it was found that PCOS is associated with oxidative stress. In present study the use of metformin alone and combination of metformin with antioxidant agents (vitamins C or E) decreases the histurism score significantly after 1, 2 and 3 months of treatment (table 2, figure 1, 2, and 3). The decrement of total serum testosterone associated with decline of histurism score in all groups and this may support the hypothesis that hirsutism in PCOS is mainly due to androgen overproduction.

In this study, it was found that the percentage of patients who get excellent effect (table 3) was more in group of metformin with vitamins C (14.2%) and E (37.5%) than that in group of metformin alone (6.25%). Moreover the worsening effect in patients who treated with metformin alone is (12.6%) while the patients who treated with metformin and antioxidant agents show no such bad effect. This may indicate that antioxidant agents participate in alleviation of hirsutism so it can be said that oxidative stress may play an important role in developing of hirsutism in PCOS. Therefore, the pathogenesis of histurism in PCOS may differ from other conditions where it may caused by the androgen overproduction in addition to oxidative stress. In conclusion, it can be said that use of antioxidant agents are of great benefit in PCOS; in addition to their excellent effect on histurism score at least they may prevent worsening effect.

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Effects of Allopurinol on Ketone Body Metabolism and Tissue Lipid Peroxidation in Alloxan Diabetes in Rats.

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ABSTRACT

The aim of the present study is to investigate whether or not xanthine oxidase (XO)–derived reactive oxygen species (ROS) may play a role in the pathogenesis of alloxan (ALX)–induced diabetes in rats using the specific XO inhibitor and hydroxyl radical scavenger, allopurinol

The involvement of oxidative stress in ALX–diabetes was assessed by the measurement of plasma and various tissues lipid peroxidation levels ( using thiobarbituric acid (TBA) reactive substances ). Furthermore, the ability of allopurinol to influence these and other biochemical parameters, including plasma and urine ketones levels were also investigated in diabetic rats.

Rats were divided into four groups: control, untreated diabetic, allopurinol – treated diabetic, and insulin – treated diabetics. At the end of the one week experimental period, blood and tissue samples were obtained from anesthetized animals for the measurement of the above – mentioned parameters.

Although the single intraperitoneal (i.p.) injection of allopurinol (25 mg/kg body wt.) 1h before or 1h after ALX injection (100 mg/kg body wt., i.p.) failed to prevent the induction of diabetes, it did lower ketonuria and the incidence of early ketosis–associated mortality in diabetic animals in comparison with non–allopurinol–treated diabetic rats. Subsequent administration of allopurinol (25 mg/kg body wt., i.p.) every 48 hr for 1 wk (i.e., 3 additional doses) also decreased plasma ketone bodies levels as well as plasma and tissue (heart, liver, kidney, pancreas) lipid peroxides levels in comparison with non–allopurinol–treated diabetic rats. Daily insulin injection (9–12 U/kg body wt., S.C.) for 1 wk period normalized all of the above–mentioned abnormalities.

The present results suggest that XO–derived ROS play a minor role (if any) in the diabetogenic effect of ALX. On the other hand, although the mechanism(s) underlying the protective effects of allopurinol on the diabetic state is presently unknown, these effects may reflect a possible association between impaired ketone body metabolism lipid peroxidation and suggest an effect of allopurinol on ketone body metabolism.

خليصة:

إن الهدف من الدراسة الحالية هو تقصي امكانية وجود دور لأنواع الأوكسجين المتغيرة المنشأة، والمتكونة من انزيم الزيت او كستيز، في أمراض السكري المحدثة بواسطة الالوكرين في الجرذان، وذلك باستخدام عقار الألوبرينول، الشبكة الخاصل لأنزيم الزيت او كستيز ككاسم جذور الهيدروكسيل. 

وتم تقسيم الالعاب التي ي︱ود الارجا التأكدKI في الجرذان المحدثة بواسطة الالوكرين من خلال قياس مستويات بير ووكسيدات الدهن في البايئا وما اعداد من الانجذام (باستخدام المواد المتفاعلة مع حمض الباربتيت ، بتضخيم الدهن). بواسطة ذلك تم تشخيص الدهن في الالوكرين في التفرع على هذا الفئة وكذلك على المعايير الميكانيكية الحياتية الأخرى. ومن ضمها مستويات الكيتونات في الالوكرين والبول في الجرذان المصابين بالسكري. وتستريح الجرذان في استعمال مجموعتين: الفئة المعوية والسكري غير المضطرب. وتتسرب الجرذان إلى الفئة المعوية والسكري المعوية بالالوكرين. يوجد ان تأثير الجرداء المتدهدة لاكسون واحد. لم يُخذ عناناء من هذه الأعراض المختارة أعلاة. وعلى الرغم من أن الكيتون، والمرة واحدة، لعقار الألوبرينول (25 ملغ/كغم وزن الجسم في البول) قبل ساعه أو بعد ساعه من حق الالوكرن (100 ملغ/كغم وزن الجسم الغامض قل في البارود) للإدأ إلى تقليل مستوي الكيتون في البارود، وكذلك إلى ملاحظة حدوث الوفيات المبكرة الدقيقة للكت بنزيف الالوكرن في الجرم). في الحيقات المعوية بالبارود البارود بالمتلازمة مع تلك غير الالوكرن. وقد أدى الحقن اللاحق، ثلاث مرات لـ الاوبرينول (25 ملغ/كغم وزن الجسم في البول، مدة كل 48 ساعه) لدءة أسبوع، كذلك لم تكن مستويات الكيتون في البارود البارود والدين انفرا. وخفض مستويات بير وكسيدات الدهن في البارود، في العديد من الانجذام (البالي، البال، البارد، الالوكرين) بالمقارنة مع تلك الالوكرن. للقياذ الأكسون والكيسي، الالوكرنول. قد أدى الحقن البارود (9-12 وحدة/كغم وزن الجسم تحت البارود) لدءة أسبوع إلى تصحيح جميع التغيرات غير الطبيعية في المعايير المذكورة أعلاه في الحيات السكري. وتشير النتائج الحالية لوجود دور مضاعف (إن وق) لانواع الأوكسجين المتغيرة المنشأة، والمعينة من انزيم الزيت أو كستيز في التأثير المحدث للسكري الالوكرنول. ومن ناحية أخرى، وعلى الرغم من عدم عصابة الأليا (الإعالة) المتناسبة عن التأثيرات المغيرة لـ الألوبرينول في الوقت الحاضر، إلا أنها تكرر ممكن امكانية وجود علاقة ما بين الخلل في العملية الإيضانية للكوتون وبين بروستيكلت في مرض السكري. وقد تشير أيضاً إلى وجود تأثير لعقار الألوبرينول على العملية الإيضانية للكوتون.

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INTRODUCTION
There is considerable evidence that xanthine oxidase (XO) system (one source of superoxide radicals in the body) may be involved in the generation of reactive oxygen species (ROS) in several conditions associated with oxidative stress, including chronic obstructive pulmonary disease (10), ischemia–reperfusion injury (2), endothelial dysfunction in type II diabetes (3). Furthermore, in vitro studies have suggested that ROS (generated during the oxidation of hypoxanthine by XO) may play a role in causing oxidative damage to rat pancreatic β–cells (4).

The present study was therefore undertaken to investigate whether or not XO–derived ROS may play a role in the pathogenesis of ALX–induced diabetes using the specific XO inhibitor (and consequently of superoxide generation) as well as hydroxyl radical scavenger, allopurinol (5). If the mechanism(s) of ROS production in ALX–diabetes is mediated, at least in part, through the enzyme XO, then allopurinol should either prevent or at least ameliorate the diabetic state.

MATERIALS and METHODS
Adult female Wistar rats (200–250 g) were housed in hanging plastic cages in a room kept at 22–25°C with a 14hr light and 10hr dark cycle. Animals were allowed free access to food and water during the entire experimental period except that of the induction of diabetes where animals were fasted for 48hr prior to the administration of alloxan.

Diabetes was induced in ether–anesthetized animals by the intraperitoneal (i.p.) injection of alloxan (Sigma Chemicals Co, USA) at a dose of 100 mg/kg body weight as a freshly prepared solution (100 mg/ml) in saline. ALX–treated animals were allowed to drink 5% glucose solution overnight to overcome drug–induced hypoglycemia (6). The diabetic state was monitored by the frequent daily testing for glucosuria (with Lilly Tes–Tape, Eli Lilly & Co, USA) and for ketonuria (using strips obtained from Ketostix, Ames Co, USA).

The following groups were studied :
1-Control rats (n = 6).
2-Untreated diabetic rats (n = 12). Rats of this group received the alkaline vehicle (0.2–0.25 ml of saline, pH 12.0) i.p. 1hr before (n = 6) or 1hr (n = 6) after ALX injection.
3-Allopurinol–treated diabetic rats (n = 12). Allopurinol (Sigma Chemical Co, USA) was prepared at a concentration of 25 mg/ml of saline pH 12.0. Rats received a single i.p. injection of 0.2–0.25 ml of alkaline vehicle to attain a final dose of 25 mg/kg body wt. 1hr before (n = 6) or 1hr (n = 6) after ALX injection. Then allopurinol was further given at 48 hr, 96 hr and 144 hr after the ALX–diabetic period. The dose of allopurinol was ¼ of that dose (100 mg/kg body wt.) reported to provide protection against oxidative–stress–induced lipid peroxidation in various tissues of rats (5).

4-Insulin–treated diabetic rats (n = 6) :
ALX–diabetic animals were injected with insulin zinc suspension (Lente MC, Novo Industri A/S, Denmark) subcutaneously at a daily dose of 9–12 U/kg body wt. immediately after detection of the diabetic state. The dosage of insulin was adjusted by daily monitoring urinary glucose and ketone levels.

At the end of the 1wk experimental period, animals were anesthetized with ether, and heparinised blood samples were obtained by cardiac puncture. Animals were killed by cardiac excision, and tissue (heart, pancreas, liver and kidney) homogenates (10% w/v) were prepared in ice–cold 50 mM Tris –0.1M EDTA buffer, pH 7.6. Plasma & tissue malondialdehyde (MDA) levels (as an index of lipid peroxidation) were determined by the thiobarbituric acid (TBA) reaction (8). The TBA–reactive substances were calculated using an extinction coefficient of MDA of 1.56 × 105.

Plasma levels of glucose, cholesterol & triglycerides as well as uric acid (to assess xanthine oxidase activity) were measured using commercial assay kits (Sigma Chemicals Co, USA). Plasma ketone bodies levels (β–hydroxybutyrate) were measured using the method of Williamson and Mallanby (9). β–hydroxybutyrate is quantitatively the predominant ketone body present in the blood in uncontrolled diabetes mellitus (10).

Statistical analyses were performed using ANOVA at significance level of P < 0.05. Further specific group differences were determined using Tukey’s test.

RESULTS
General Features of Diabetes :
The administration of the alkaline vehicle (whether given 1hr before or 1hr after ALX injection) did not prevent the incidence of diabetes, and produced comparable picture in
In regard to degree of ketosis and the early mortality among diabetic rats.

Induction of diabetes was confirmed by the presence of glucosuria and ketonuria within 24 after ALX injection. During the first week of diabetes, 6 animals died in ketosis and diabetic coma, which started as early as 48 hr after induction of diabetes.

Animals which survived this period (6/12) also showed hyperglycemia, hypertriglyceridemia, and ketonemia (Table 1) at the end of 1 wk period. However, levels of plasma cholesterol and uric acid were not changed during this period (Table 2). Plasma and tissue lipid peroxide levels were also elevated in ALX–diabetic rats (Table 3).

**Effect of Allopurinol Treatment**

Although the single i.p. injection of allopurinol (25 mg/kg body wt.) given 1hr prior to or 1hr post ALX administration did not prevent the induction of diabetes, it did lower the incidence of early ketosis–associated mortality in these animals. Unlike the high mortality in non–allopurinol–treated diabetic animals, only 2 of the 12 allopurinol–treated diabetic rats died in ketosis and coma (Table 1). The remaining 10 animals showed glucosuria and slight ketonuria. Treatment with allopurinol for 1 wk period did also lower the associated ketonemia (Table 1) as well as the lipid peroxide levels in plasma and tissues of allopurinol–treated diabetic rats compared to the non–treated diabetics (Table 3).

**Effect of Insulin Treatment**

Insulin treatment (9 – 12 U/kg body wt/day) started immediately after detection of diabetes prevented the ketosis–associated coma and mortality, and normalized all of the above–mentioned abnormalities (Table 1–3).

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**Table 1: Plasma and urine ketone body levels and mortality rate in control, untreated–ALX diabetic, allopurinol–treated–ALX diabetic, and insulin treated–ALX diabetic rats after 1 week of diabetes**

<table>
<thead>
<tr>
<th></th>
<th>Plasma ketones (B-hydroxybutyrate) (µmol/L)</th>
<th>Urine ketones* (acetoacetate)</th>
<th>Number of animals died with diabetic coma/1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>0.68 ± 0.29 a</td>
<td>(-)</td>
<td>-</td>
</tr>
<tr>
<td>Untreated–diabetic (n = 12)</td>
<td>9.20 ± 3.60 b</td>
<td>(+ + +)</td>
<td>6/12 animals (50%)</td>
</tr>
<tr>
<td>Allopurinol–treated diabetic (n = 12)</td>
<td>3.54 ± 2.02 c</td>
<td>2 (+ +)</td>
<td>2/12 animals (17%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 ( –)</td>
<td></td>
</tr>
<tr>
<td>Insulin–treated diabetic (n = 6)</td>
<td>0.98 ± 0.35 a</td>
<td>(-)</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD.

- Values with different letters are significantly different (P < 0.05).

* Urine ketone body levels were qualitatively measured using ketostix strips obtained from Ames Co, USA, as described in material and methods.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt. (g)</th>
<th>Plasma level (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>231 ± 24</td>
<td>146 ± 9 *</td>
</tr>
<tr>
<td>Untreated–diabetic (n = 6)</td>
<td>219 ± 26</td>
<td>478±75 b</td>
</tr>
<tr>
<td>Allopurinol–treated diabetic (n = 10)</td>
<td>228 ± 18</td>
<td>497 ± 120 b</td>
</tr>
<tr>
<td>Insulin–treated diabetic (n = 6)</td>
<td>238 ± 32</td>
<td>109±30 a</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SD.
- Within each column, values with different letters are significantly different (P < 0.05).


<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma n mol/ml</th>
<th>TBA – Reactive substances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td>Control (n = 6)</td>
<td>1.44±0.32 a</td>
<td>178 ± 25 a</td>
</tr>
<tr>
<td>Untreated–diabetic (n = 6)</td>
<td>5.93±2.80 b</td>
<td>600 ± 75 b</td>
</tr>
<tr>
<td>Allopurinol–treated diabetic (n = 10)</td>
<td>2.28±1.32 c</td>
<td>351 ± 88 c</td>
</tr>
<tr>
<td>Insulin–treated diabetic (n = 6)</td>
<td>1.65±0.54 a</td>
<td>156 ± 13 a</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± SD.
- Within each column, values with different letters are significantly different (P < 0.05).
DISCUSSION

The diabetic state produced in rats during the first week following ALX injection was confirmed by glucosuria, ketonuria, hyperglycemia and ketonemia. The diabetic state was also accompanied by elevated plasma and tissue lipid peroxides levels as well as increased incidence of early ketosis – related mortality.

The ability of insulin therapy to completely reverse these changes suggests that they are related to the diabetic state in this strictly insulin–dependent model of experimental diabetes (6).

In an attempt to investigate whether or not allopurinol can prevent the induction of diabetes, the single i.p. administration of 25 mg/kg body wt. failed to prevent the induction of diabetes, whether it was administrated 1 hr before or 1 hr after ALX injection. This fact is suggestive of a minor role (if any) of XO – generated ROS in the diabetogenic action of ALX, and disagrees with the conclusion reached by Heller et al. (3) who suggested that ROS generated by XO are responsible for the destruction of insulin – producing β-cells during insulits.

However, the single allopurinol administration was able not only to lower ketonuria, but also the incidence of early ketosis – associated mortality in diabetic rats, a fact suggestive of a possible effect on ketone body metabolism. Furthermore, preliminary experiments revealed that after a single i.p. administration of allopurinol, urinary ketone bodies start to increase (after the initial decline in level) again within 50–60 hr in allopurinol–treated diabetic animals: and by approximately 80 hr of diabetes no significant differences could be observed between allopurinol–treated and non–allopurinol–treated diabetic animals. This finding, suggests the involvement of allopurinol and/or its metabolite alloxanthine in these protective effects, given that the half–life of allopurinol is 2–3 hr and that of alloxanthine is 18–30 hr (11). Accordingly, the present experiment also applied repeated administration of allopurinol every 48 hr for 6 days. Interestingly, these repeated administration of allopurinol did lower ketone body levels in both urine and plasma and the lowered mortality rate (due to the first single dose) was maintained till the end of experimental period (1 week).

It is important to point out that although alkali treatment has been recommended to correct diabetic ketoacidosis (12), the possibility that the ability of allopurinol to lower ketonuria is in fact attributable to the high alkalinity of the dissolving vehicle (pH 12) rather than to a direct effect of allopurinol per se has been ruled out. Diabetic animals receiving the same alkaline vehicle failed to demonstrate similar protective effects.

In a computer med–line search of the literature from 1970 –2002, no comparable reports concerning the effect of allopurinol treatment on ketone body metabolism in diabetes were found; therefore, the mechanism(s) responsible for the protective effects of allopurinol against the increased levels of ketone bodies remain(s) to be investigated in further studies. It is possible that allopurinol exerts its effects through an inhibition of XO; however, the fact that allopurinol treatment did not lower plasma uric acid in ALX –diabetic rats makes it an unlikely possibility.

The ability of allopurinol to lower ketonuria and ketonemia could be explained through either increasing ketone bodies metabolism by various tissues and/or decreasing their synthesis via affecting deacylase or β–hydroxy–β–methylglutaryl–CoA formation (13). It is unlikely that allopurinol might have enhanced endogenous insulin release because other insulin–dependent abnormalities were not corrected by allopurinol treatment.

The ability of allopurinol to lower ROS–induced lipid peroxidation provides another evidence for the potential beneficial effect of allopurinol treatment on tissue antioxidant defenses against diabetes–induced increased oxidative stress. It further supports the findings of other studies which reported that allopurinol lowered MDA levels in plasma of patients with type II diabetes with mild hypertension (10) and in various tissues of rats exposed to oxidative stress induced by cypermethrin (7) or by experimental acute pancreatitis (10). Furthermore, the possibilities of a direct effect of allopurinol and alloxanthine on ketone body metabolism and lipid peroxidation independently of inhibition of XO, or via scavenging the powerful hydroxyl radicals (14), or may involve generalized alterations in tissue antioxidant status (2) can not be ruled out at present. Much more work must be done to assess whether there is any relationship between lipid peroxidation and ketosis in ALX diabetes and further investigate the protective effects of ALP in this regard.

In conclusion, the present study suggests that under the conditions tested and in the doses and duration of treatment used, allopurinol through its probable or apparent ability to inhibit generation of ROS (especially hydroxyl radical scavenging) rather than XO inhibition.
appears to have a protective effect on both lipid peroxidation and ketogenesis and consequently early mortality in ALX–diabetes.

REFERENCES


Effect of Nitrogen and Phosphorus Levels on Yield,
Concentration, Physical and Chemical Properties of Dill Seed Oil
Fadhil H Al-Saha*, Madeha H Al-Samara** (1), Muna J Al-Ndawi**

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ABSTRACT
To test the effect of 4 levels of nitrogen (i.e. 0, 45, 90 and 135 Kg N ha⁻¹) as urea (46% N) and 3 levels of phosphorus (i.e. 0, 17.5 and 35 Kg P ha⁻¹) as triple super phosphate (21.8% P) on yield and concentration of dill (Anethum graveolens L. local cultivar) seed oil this experiment was carried out during winter season of 1999 - 2000 at the experimental field of Agriculture College, Abu-Ghraiab.

Both fertilizers were applied in two equal splits, first at seeds sowing and the second was added one month after emergence. Dried and ground seed samples were subjected to water distillation for extraction of volatile oils.

Result indicated that fertilization of dill plants with 90 Kg N + 35 Kg P ha⁻¹ produced highest oil yield (32.19 ℓ ha⁻¹) and concentration (3.60%) with better quality. GLC analysis indicated that dill seed oil contain 27 volatile compounds, 15 were identified and the major constituents were Carvone, Limonene and α-Phellandrene. Nitrogen and phosphorus fertilization increased the concentration of all identified constituents of dill seed oil.

Nitrogen and phosphorus fertilization increased yield and concentration of dill seed oil. Moreover, physical properties of the oil were also improved by N and P fertilization. GLC analysis showed that Carvone, Limonene and α-Phellandrene are the major constituents of dill seed oil and could be increased by N and P application.

Key words : Volatile compounds, Carvone, Limonene, α-Phellandrene.

INTRODUCTION
Dill (Anethum graveolens L.) belong to Umbelliferae is one of nutritional and medicinal vegetables (1). Dried leaves and ground seeds are used as flavour additives to the scald meat and cheeses (2), and to pickle cucumbers to improve taste and maintain good quality (3). Dill seed oil is used for medical purposes as carminative, in flatulence of children, antispasmodic, stop children’s stomach pain, and aid lactation (4). It has also been used for lowering blood hypertension and blood sugar level in diabetics (5).

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**Technical Institute, Al-Mansour, Baghdad-Iraq
(1) Part of M.Sc. thesis of the second author.
Nitrogen fertilization increased seed oil yield and changed its chemical constituents. However, levels of N fertilization higher than 120 Kg N ha\(^{-1}\) decreased oil concentration in dill seeds. Singh and Mahey also found that maximum seed oil concentration was reached when dill plants were fertilized with 120 Kg N + 17.5 Kg P ha\(^{-1}\).

Dill oil density usually is lower than the density of water so it floating as oily layer on water surface during extraction, and affected by sowing dates but not by N or P fertilization, whereas, refractive index of the oil was increased by N and P fertilizers. The objective of the present work was to examine the interactive effect of nitrogen and phosphorus application on dill seed oil yield, concentration and volatile oil constituents.

**MATERIALS and METHODS**

**Experiment layout**

A field experiment was carried out during the winter season of 1999-2000 at the experimental field of Horticulture Department, Agriculture College University of Baghdad. Dill seeds (local cultivar) were sown on 1\(^{st}\) of October 1999 in rows 15 cm apart in plots (2.5 m\(^2\)) at a rate of 2.5 Kg seeds ha\(^{-1}\). Treatments included 4 levels of nitrogen as urea (46% N) \((0, 45, 90, 135 \text{ Kg N ha}^{-1})\) referred to as N\(_0\), N\(_1\), N\(_2\) and N\(_3\), respectively, and 3 levels of phosphorus as triple superphosphate (21.8% P) \((0, 17.5, 34 \text{ Kg P ha}^{-1})\) referred to as P\(_0\), P\(_1\) and P\(_2\), respectively. Fertilizers were applied in two equal splits, first on seed sowing and the second was applied one month after emergence. Fruits were collected progressively when the colour turned brownish yellow and air dried on 15\(^{th}\) of May 2000.

**Oil Extraction**

Ground seeds were water distilled according to British pharmacopoeia as described by ASTA. Clevenger apparatus was connected to rotary evaporator flask where 25g of seeds powder were distilled with 250 ml of distilled water, and distillation continued for 2 hr. Oil extract was transferred to a separatory funnel, and 30ml of diethyl ether (C\(_5\)H\(_{12}\)O) was added. The mixture was hand shaken and left to settle, a layer of oil and diethyl ether floated on the top of the extract, collected and this procedure was repeated three times for each sample. 3 – 5 g of calcium sulfate were added to the oil – ether mixture to absorb remaining water droplets. Ether was evaporated in rotary evaporator under vacuum at 37 c°, the oil transferred to dark brown color vials and stored in refrigerator (4 ± 1 c°).

**Physical Properties of Dill Seed Oil**

Extracted seed oil physical properties of different treatment samples were determined according to Guenther those included specific gravity and density. Refractive index was determined by Abbe Refractometer (Abbe Type Universal, Haensch, Schmidt Co.). Determination of physical properties were performed at 20 c°.

**Chemical Analysis**

Chemical analysis of dill seed oil constituent was performed by Gas-liquid chromatography (Ge-9A Model, Shimazu, Osaka, Japan) by direct injection onto a 30m × 0.25mm column packed with 5% polydiphenyl siloxane and 95% dimethyl siloxane on SPB-5. Separation conditions were as follows: carrier as (helium) at flow rate = 25 ml min\(^{-1}\), injection port temperature, detector (Flame ionization detector, FID) temperature, and oven temperature was 220 c°. Oil constituents were identified as compared to a number of essential oils standards.

**Statistical Design and Analysis**

A split-plot experiment was used where nitrogen levels were represented by main plots and phosphorus levels as sub-plots with three replicates. Results were subjected to the analysis of variance and least significant differences (LSD P = 0.05) was used to compare the difference between treatments.

**RESULTS and DISCUSSION**

Oil yield and content of dill seeds oil were increased markedly by increasing nitrogen levels up to 90 Kg N ha\(^{-1}\) (N\(_2\)) (Table 1). The percent increases in oil yield was 114.5% whereas for oil concentration was only 49.4% when comparing N\(_2\) to N\(_0\). These increases may accounted for the increases in vegetative growth mainly leaf area which may reflected on dry matter and secondary metabolites production. However, increasing nitrogen levels up to 135 Kg ha\(^{-1}\) resulted in a significant reduction in both yield and concentration of oil in dill seeds. These results may suggest that optimum level of nitrogen fertilization for highest yield and...
concentration of dill seed oil is 90 Kg ha⁻¹. Singh and Randhawa (10) had also found that fertilization of dill plant with this same level of nitrogen produced the highest yield and concentration of oil in the seeds, and increasing the N level up to 120 Kg ha⁻¹ caused a significant reduction in both yield and concentration of the seeds oil.

Increasing phosphorus level, from P₀ to P₂ had also increased yield and concentration of dill seeds oil but to lesser extent as compared to the effects of nitrogen levels increases, where the percent increases were 13.7% and 4.8% for the oil yield and concentration respectively. These differences in the effects between N and P fertilization could be attributed to the magnitude effect of either nutrient in vegetative growth. The interaction was significant and the highest oil yield (32.19 ℓ ha⁻¹) and concentration (3.6%) was produced when dill plants were fertilized with 90 Kg N ha⁻¹ + 35 Kg P ha⁻¹. Similar effects of N + K on dill seeds oil yield and content was found by Singh and Mahey (12).

### Physical Properties of the Dill Seed Oil

Nitrogen application to dill plants had no marked effects on seed oil specific gravity, density and refractive index (Table 1). Increasing phosphorus level also was not effective in oil specific gravity and density, while refractive index was slightly increased (but significant) as phosphorus level increased.

### Table 1. Effect of nitrogen and phosphorus levels on dill seed oil yield, concentration and physical properties.

<table>
<thead>
<tr>
<th>Fertilizer Level</th>
<th>Seed oil yield (ℓ ha⁻¹)</th>
<th>Seed oil content (%)</th>
<th>Oil specific gravity</th>
<th>Oil density (mg µ ℓ⁻¹)</th>
<th>Refractive index</th>
</tr>
</thead>
<tbody>
<tr>
<td>N levels N₀</td>
<td>14.28</td>
<td>2.39</td>
<td>0.933</td>
<td>0.873</td>
<td>1.487</td>
</tr>
<tr>
<td>N₁</td>
<td>25.42</td>
<td>3.19</td>
<td>0.942</td>
<td>0.880</td>
<td>1.488</td>
</tr>
<tr>
<td>N₂</td>
<td>30.63</td>
<td>3.57</td>
<td>0.943</td>
<td>0.881</td>
<td>1.488</td>
</tr>
<tr>
<td>N₃</td>
<td>22.08</td>
<td>2.69</td>
<td>0.940</td>
<td>0.877</td>
<td>1.489</td>
</tr>
<tr>
<td>L.S.D₀.₀₅</td>
<td>1.715</td>
<td>0.004</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
</tr>
<tr>
<td>P levels P₀</td>
<td>21.47</td>
<td>2.89</td>
<td>0.937</td>
<td>0.876</td>
<td>1.486</td>
</tr>
<tr>
<td>P₁</td>
<td>23.41</td>
<td>2.97</td>
<td>0.944</td>
<td>0.882</td>
<td>1.487</td>
</tr>
<tr>
<td>P₂</td>
<td>24.42</td>
<td>3.03</td>
<td>0.938</td>
<td>0.877</td>
<td>1.489</td>
</tr>
<tr>
<td>L.S.D₀.₀₅</td>
<td>1.485</td>
<td>0.004</td>
<td>n.s</td>
<td>n.s</td>
<td>0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N₀P₀</td>
<td>13.72</td>
<td>2.36</td>
<td>0.921</td>
<td>0.861</td>
<td>1.485</td>
</tr>
<tr>
<td>N₀P₁</td>
<td>14.26</td>
<td>2.40</td>
<td>0.947</td>
<td>0.885</td>
<td>1.487</td>
</tr>
<tr>
<td>N₀P₂</td>
<td>14.84</td>
<td>2.43</td>
<td>0.933</td>
<td>0.872</td>
<td>1.489</td>
</tr>
<tr>
<td>N₀P₀</td>
<td>22.26</td>
<td>3.06</td>
<td>0.948</td>
<td>0.887</td>
<td>1.486</td>
</tr>
<tr>
<td>N₀P₁</td>
<td>25.77</td>
<td>3.18</td>
<td>0.942</td>
<td>0.881</td>
<td>1.488</td>
</tr>
<tr>
<td>N₀P₂</td>
<td>28.24</td>
<td>3.33</td>
<td>0.934</td>
<td>0.873</td>
<td>1.489</td>
</tr>
<tr>
<td>N₀P₀</td>
<td>28.79</td>
<td>3.53</td>
<td>0.934</td>
<td>0.874</td>
<td>1.486</td>
</tr>
<tr>
<td>N₀P₁</td>
<td>30.90</td>
<td>3.57</td>
<td>0.950</td>
<td>0.887</td>
<td>1.488</td>
</tr>
<tr>
<td>N₀P₂</td>
<td>32.19</td>
<td>3.60</td>
<td>0.945</td>
<td>0.888</td>
<td>1.489</td>
</tr>
<tr>
<td>N₀P₀</td>
<td>21.12</td>
<td>2.61</td>
<td>0.941</td>
<td>0.880</td>
<td>1.486</td>
</tr>
<tr>
<td>N₀P₁</td>
<td>22.71</td>
<td>2.71</td>
<td>0.938</td>
<td>0.877</td>
<td>1.487</td>
</tr>
<tr>
<td>N₀P₂</td>
<td>24.42</td>
<td>2.74</td>
<td>0.942</td>
<td>0.880</td>
<td>1.488</td>
</tr>
<tr>
<td>L.S.D₀.₀₅</td>
<td>5.50</td>
<td>0.008</td>
<td>0.014</td>
<td>0.013</td>
<td>n.s</td>
</tr>
</tbody>
</table>

However, the interaction effect of N and P fertilization was significant and the highest specific gravity and density of the oil was noticed when plants were fertilization with N₀P₁ and N₀P₂ respectively, whereas refractive index was not affected significantly by the interaction treatments. Changes in physical properties of the oil by N and P fertilization could be due to the effect of both nutrients on oil oxygenous compounds such as Carvone, Linalool, Eugenol, α-Tujone and Citronellol (19). Positive correlation was found between phosphorus content of the vegetative parts and oil specific gravity (r = 0.433*), density (r = 0.425*) and refractive index (r = 0.784**). These relationships could be explained on the basis of the effect of phosphorus on essential oil solid constituents (oxygenous compounds) (19). These findings are
in accordance with Randhawa et al. (11) who concluded that physical properties of dill seed oil is not sensitive to N or P fertilization probably because of the difference in cultivars used and/or predominant environmental conditions. Essential oils with high physical properties especially refractive index and density regarded as high quality.

Seed Oil Constituents Analysis by GLC

Analysis of dill seed oil showed that it contained 27 volatile oil components, 15 were identified and the rest were not because of lack of standards. These 15 compounds could be classified to three groups according to their concentrations, those are high (Carvone, Limonene, and α-Phellandrene), medium (Camphene, Linalool, Citronellol, Myrcene, Myristicin, Terpenolene, Terpene and Caryophyllene), and low (α-Thujone, β-Cymene, α-Pinene and Eugenol) (Table 2). Phellandrene and to some extent Limonene (monocyclic terpenic compounds) are the main constituents for odor and flavor of dill seed oil (16).

Increasing nitrogen levels increased the concentration of all identified volatile compounds. However, these increases were not linear as nitrogen level increased, where the concentration of all oil constituents were notably dropped when nitrogen application rose up to 135 Kg ha⁻¹ (N₃). Concentration reduction could be attributed to the production of other metabolites such as pigments, hormones ....... etc., in addition to the dilution effect.

Increasing phosphorus levels gradually increased the concentrations of all identified volatile compounds of dill seed oil. Comparing the effect of N and P levels concentrations of Carvone, Limonene and α-Phellandrene, Which are the major constituents, phosphorus was more effective than nitrogen, where the percent increases due the N₂ or P₂ were 17.30% and 49.35% , 59.79% and 230.22% , and 225.63% and 378.81% for the three compounds respectively. However, Camphene, Terpenolene and Myrcene (medium concentration group) and β-Cymene and α-Pinene (low concentration group) had been affected drastically by increasing nitrogen level to 90 Kg ha⁻¹ as compared to the effect of increased level of phosphorus to 35 Kg ha⁻¹, where the percent increases were 225.29% and 91.12%, 345.77% and 57.86%, 182.62% and 147.44%, 1921.28% and 77.08%, and 968.25% and 118.61% respectively. These variations could be due to the rule of either nutrient in metabolism and synthesis of these compounds. These results reveal that those nutrients may have different mode of action in the biosynthesis of these compounds.

<table>
<thead>
<tr>
<th>Table 2. Effect of nitrogen and phosphorus levels on dill seed oil constituents concentration (mg Kg seed⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Compounds</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Carvone</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Limonene</td>
</tr>
<tr>
<td>α-Phellandrene</td>
</tr>
<tr>
<td>Camphene</td>
</tr>
<tr>
<td>Linalool</td>
</tr>
<tr>
<td>Eugenol</td>
</tr>
<tr>
<td>α-Thujone</td>
</tr>
<tr>
<td>Citronellol</td>
</tr>
<tr>
<td>Myrcene</td>
</tr>
<tr>
<td>β-Cymene</td>
</tr>
<tr>
<td>Myristicin</td>
</tr>
<tr>
<td>α-Pinene</td>
</tr>
<tr>
<td>Terpenolene</td>
</tr>
<tr>
<td>Terpine</td>
</tr>
<tr>
<td>Caryophyllene</td>
</tr>
</tbody>
</table>
Concentration of most identified seed oil constituents were the highest when dill plants fertilized with 90 Kg N + 35 Kg P ha-1 (Table 3). These results could be due to the optimum balance of N and P fertilization for increasing the concentration of seed oil constituents.

Table 3. Interactive effect of nitrogen and phosphorus levels on dill seed oil constituents concentration (mg Kg seeds$^{-1}$)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>N0</th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P0</td>
<td>P1</td>
<td>P2</td>
<td>P0</td>
</tr>
<tr>
<td>Carvone</td>
<td>235.6</td>
<td>469.1</td>
<td>485.2</td>
<td>263.1</td>
</tr>
<tr>
<td>Limonene</td>
<td>24.6</td>
<td>78.2</td>
<td>102.9</td>
<td>45.5</td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>26.8</td>
<td>75.0</td>
<td>60.5</td>
<td>42.5</td>
</tr>
<tr>
<td>Camphene</td>
<td>12.6</td>
<td>23.8</td>
<td>38.0</td>
<td>38.5</td>
</tr>
<tr>
<td>Linalool</td>
<td>11.5</td>
<td>25.5</td>
<td>39.1</td>
<td>27.8</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.791</td>
<td>1.95</td>
<td>2.89</td>
<td>1.52</td>
</tr>
<tr>
<td>α-Thujone</td>
<td>0.405</td>
<td>0.856</td>
<td>1.14</td>
<td>0.646</td>
</tr>
<tr>
<td>Citronellol</td>
<td>0.415</td>
<td>8.88</td>
<td>19.9</td>
<td>0.539</td>
</tr>
<tr>
<td>Myrcene</td>
<td>1.21</td>
<td>6.86</td>
<td>16.6</td>
<td>7.33</td>
</tr>
<tr>
<td>β-Cymene</td>
<td>0.227</td>
<td>1.25</td>
<td>1.49</td>
<td>9.42</td>
</tr>
<tr>
<td>Myristicin</td>
<td>6.51</td>
<td>7.38</td>
<td>8.55</td>
<td>11.0</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>0.375</td>
<td>0.658</td>
<td>0.870</td>
<td>2.42</td>
</tr>
<tr>
<td>Terpenolene</td>
<td>2.61</td>
<td>4.04</td>
<td>4.71</td>
<td>4.74</td>
</tr>
<tr>
<td>Terpineen</td>
<td>13.4</td>
<td>16.2</td>
<td>22.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Caryophyllene</td>
<td>0.586</td>
<td>14.7</td>
<td>43.5</td>
<td>2.41</td>
</tr>
</tbody>
</table>

CONCLUSIONS

Nitrogen and phosphorus fertilization increased yield and concentration of dill seed oil. Moreover, physical properties of the oil were also improved by N and P fertilization. GLC analysis showed that Carvone, Limonene and α-Phellandrene are the major constituents of dill seed oil and could be increased by N and P application.

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In Vitro Release Study on Capsules and Tablets Containing Enteric-Coated Granules Prepared by Wet Granulation
Eman B. H. Al-Khedairy*

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ABSTRACT
Wet granulation method was used instead of conventional pan coating or fluidized-bed coating technique to prepare enteric-coated diclofenac sodium granules, using ethanolic solution of Eudragit\textsuperscript{TM} L100 as coating, binding and granulating agent. Addition of PEG400 or di-n-butyl phthalate as a plasticizer was found to improve the enteric property of the coat.
Part of the resulted granules was filled in hard gelatin capsules (size 0), while the other part was compressed into tablets with and without disintegrant.
The release profile of these two dosage forms in 0.1N HCl (pH 1.2) for 2 hours, and in phosphate buffer (pH 6.8) for 45 minutes as well as the release kinetic were compared with that of the enteric film coated Voltadin\textsuperscript{R} SDI tablets.
The results of this study show that, the prepared dosage forms have a good enteric property, with faster release of drug from encapsulated enteric-coated granules in comparison with compressed tablets.

INTRODUCTION
Enteric coated granules encapsulated in hard gelatin capsules as a pharmaceutical dosage form were used to get products with fast onset of absorption and better pharmacokinetic properties\cite{1-3}, since this dosage form is less influenced by food intake due to faster gastric emptying of granules after dissolving of hard gelatin shell compared with the retention of relatively large enteric coated tablet in the stomach.
In addition, patients preferred the easily swallowed gelatin capsules on tablets\cite{4}.
Different techniques were used to prepare enteric-coated granules or pellets, such as pan coating and fluidized bed coating apparatus\cite{5-6}.
The aim of this study is to investigate whether enteric-coated granules could be prepared by ordinary wet granulation method using ethanolic solution of Eudragit\textsuperscript{TM} L100 as coating and granulating agent.

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Method:
- **Preparation of Enteric-Coated Granules:**
  Diclofenac sodium was mixed with lactose and the powder mass was moistened with 2% ethanolic solution of Eudragit® L100 as a granulating, binding and enteric coating agent until the proper consistency was obtained. The amount of enteric polymer was about 1.25% based on the total weight of the powder mixture. The moist mass was then granulated by passing through a 0.8 mm mesh size sieve. The granules were then dried on trays. Part of the resulted granules was filled in hard gelatin capsules size 0 (25mg/capsule) while the other part was compressed into tablets (25mg/tablet).

- **Dissolution:**
  The dissolution characteristics of the encapsulated enteric-coated granules, the compressed tablets from the prepared granules and Voltadin® SDI tablets as a reference were studied using USP XXIV method for enteric coated products at 50 r.p.m and constant temperature (37±0.5 °C). One capsule or tablet of each was placed in 750 ml 0.1 N HCl (pH 1.2) and samples were taken for 2 hours, followed by addition of 250 ml of 0.2M trisodium phosphate to the same jar to get phosphate buffer of pH 6.8, then sampling were continued for 45 minutes.
  Samples were taken at certain time intervals and assayed for diclofenac sodium spectrophotometrically at its λmax 273 nm for the acid medium and at λmax 276 nm for the buffer medium.

- **Factors Affecting the Preparation:**
  1. **Effect of Addition of Plasticizer:**
     Two different types of plasticizers were used to study their effect on improving the coating property of Eudragit® L100. A water-soluble type (PEG 400) and non water-soluble (Di-n-butyl phthalate), 10% of either type of plasticizer calculated on the amount of dry lacquer substance was added to the coating solution.
  2. **Effect of Compressing the Granules:**
     Part of the resulted enteric-coated granules was compressed into tablets. The results of dissolution of these tablets were compared with that of encapsulated enteric-coated granules.

3. **Effect of Addition of Disintegrant:**
   10% Avicel PH 102 calculated on the total amount of granules was added extragranularly to the enteric coated granules before compression. The results of dissolution were compared with those of encapsulated enteric-coated granules as well as with tablets prepared from the same granules but without disintegrant.

**RESULTS and DISCUSSION**
- **Dissolution in Acid Medium:**
  1. **Effect of Addition of Plasticizer:**
     The effect of plasticizer was studied by comparing the release of drug from the encapsulated granules coated with Eudragit® L100 in acid medium with and without addition of plasticizer.
     The results show that 15% of the drug was released from the coated granules in the absence of plasticizer in the coating solution, which is not accepted according to the USP requirements for the enteric-coated preparations, which state that not more than 10% of drug dissolves at the end of 2 hours.
     Addition of either PEG 400 or di-n-butyl phthalate to the coating solution decreased the release of the drug after 2 hours of dissolution in acid medium to 6.2% and 8.5% respectively in comparison with 10% released from the reference Voltadin® SDI tablets (Fig. 1), which are all acceptable.
     These results indicate that addition of either of the plasticizer can improve the enteric property of the coating polymer which may be due to increasing the mobility of the chain of the polymer at the surface of the granules, so they facilitate the filming property of the polymer, but since di-n-butyl phthalate is non water soluble, it will decrease the permeability of film to moisture and enhance the stability of the product, so it is preferred for the preparation of this product on PEG 400 therefore the granules prepared with PEG 400 were neglected.
  2. **Effect of Compressing the Granules:**
     Compressing of the granules prepared using di-n-butyl phthalate as plasticizer for the coating material to tablets with and without addition of disintegrant, resulted in decreasing the release of drug in the acid medium of dissolution to 7.4% and 6.3% respectively in comparison with the encapsulated granules (Fig. 1). The decrease in the release of drug may be due to the smaller surface area of the tablets compared to the granules, while the slight difference in the release between the two tablet preparations, may be due to the effect of disintegrant.
Acid medium
Buffer medium
pH(1.2) medium pH(6.8)

Fig. (1) Dissolution profile of encapsulated enteric-coated granules Prepared with and without addition of disintegrant in comparison with Voltadin(R) SDI tablets

- Dissolution in Buffer Medium:
  As shown in Fig. 1, higher and faster release was obtained from encapsulated enteric-coated granules in comparison with Voltadin(R)SDI tablets and tablets prepared with and without addition of disintegrant, where about 90% of the drug was released from these granules within 20 minutes (8) compared to 72%, 57.5%, and 49.7% from the other tablets, respectively. This high and fast release of drug is mainly due to the larger surface area of the granules compared to the tablets.

  In addition, both the encapsulated enteric-coated granules and tablets containing disintegrant as well as Voltadin(R)SDI tablets met the USP specifications for the release of drug in buffer solution (not less than 75% of drug dissolves at the end of 45 minutes) (10) in contrast to tablets without disintegrant.

Kinetics of Dissolution:
The release characteristics of the drug from encapsulated enteric-coated granules and tablets prepared from these granules in presence of disintegrant as well as that of Voltadin(R) SDI tablets, follow first order kinetics, since plotting the logarithm of the percent remaining versus time gave straight lines with good correlation (r-value range from -0.94 to -1.01) for the acid medium and the two phases in the buffer medium of dissolution as shown in Fig.2.

Fig. (2) Plot of the log % remaining versus time for the release of drug from encapsulated enteric-coated granules and tablets prepared with addition of disintegrant in comparison with Voltadin(R) SDI tablets.

The small value of the release rate constant in the acid medium (pH 1.2) (Table 1) gives an indication that the coating material (Eudragit<sup>TM</sup> L100) when used as granulating and binding agent is effective in preventing the release of drug in acidic pH. However, the small and slow release of drug at this pH may be either due to the presence of small-uncoated drug or due to the release of drug through the coating and its possible discontinuities (15).

As the pH of the dissolution medium reaches the level critical for the coating pH 6.8 (buffer medium) the film starts to dissolve, thereby increasing the release of drug (9, 15) as shown in Fig.2 and Table 1, in which there is a great increase in the release rate constants of the two dosage forms in the buffer medium mainly at the first phase (more than 100 times) compared to that at acid medium.

In addition, the results of dissolution at the buffer medium give an indication that the release of drug is affected by the dosage form...
as well as by the surface area, since the release rate constants for the encapsulated enteric-coated granules are higher than that of compressed tablets.

Table (1)
Release rate constants (K) min⁻¹ of the Prepared Dosage Forms in Comparison with Voltadin(R) SDI Tablets

<table>
<thead>
<tr>
<th>pH The Dissolution Medium</th>
<th>Encapsulated Enteric-coated Granules using di-N-Butyl Phthalate as Plasticizer</th>
<th>Tablets with Disintegrant</th>
<th>Voltadin(R) SDI Tablets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>7.67×10⁻²</td>
<td>5.75×10⁻²</td>
<td>8.3×10⁻³</td>
</tr>
<tr>
<td>6.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K₁</td>
<td>0.309</td>
<td>0.143</td>
<td>0.098</td>
</tr>
<tr>
<td>K₂</td>
<td>0.031</td>
<td>0.020</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Abbreviations:-
K₁: refers to the dissolution rate constant for the first phase in the buffer medium
K₂: refers to the dissolution rate constant for the second phase in the buffer medium

References:-
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Gender Differences of Serum Leptin Hormone Levels in Iraqi Population
Besma I.Mustafa
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ABSTRACT
To evaluate and compare serum Leptin hormone level between Iraqi male & female and the relation between this hormone & BMI in these two groups.
A total of 44 normal male & female subjects were included in this study {Group 1 : 22 female }, { Group 2 : 22 male}.
Serum Leptin hormone ,BMI &fasting blood glucose were measured for both groups.
Serum Leptin level in group 1 was (8.82 ± 2.9 μg/L) where as in group 2 it was (4.65 ± 3.2 μg/L) . These changes were statistically significant. Fasting blood glucose levels were technically within the normal value (116.43 ± 3.4mg /dl) for group 1 & (118.52 ± 2.9 mg /dl) for group 2 . BMI levels were comparable in both groups during the study, with slight elevation in group 1 [ 24 ± 1.73 kg /m² for group 1 & 23 ± 1.98 kg /m²for group 2 ] which within the acceptable limit as far as safety concern.
Leptin, an adipocyte-derived hormone known to play an important role in body weight regulation, the result of this study shows that Leptin is presented differentially in Iraqi men and women ; in which it is significantly higher in women than in men serum . These observations are potentially important for the understanding of differences between men and women in regulation of food intake, weight gain, and body fat distribution.The relation between Leptin & the BMI in male and female in this study may open another approach for this hormone to be involved in –fertility & in pubertal development.

الخلاصة
لعرض قياس ومقارنة هرمون leptin (Leptin) بين الذكور والإناث في العراق وعلاقة مستوى هرمون المنكرو مع نسبة كتلة الجسم BMI في كل الجنسين.
شملت الدورة 44 شخصاً، تم تقسيمهم إلى مجموعتين: الأولى ضمت 22 أنثى والمجموعة الثانية ضمت 22 ذكرًا. تم قياس مستويات هرمون leptin ونسبة كتلة الجسم ومستوى سكر الدم في حالة الصيام لكل المجموعة. أظهرت نتائج قياس هرمون leptin في المجموعة الأولى 8.82 ± 2.9 ميكرونغرام/لتر، بينما كان مستوى هرمون leptin في المجموعة الثانية 4.65 ± 3.2 ميكرونغرام/لتر، حيث أن الفرق كان معيناً بصورة واضحة. وكان مستوى سكر الدم ضمن القيم الطبيعية: 116.43 ± 3.4 ملغ/100مل للمجموعة الأولى و118.52 ± 2.9 ملغ/100مل للمجموعة الثانية. وكانت نسبة كتلة الجسم مترافقة بين المجموعتين مع وجود ارتفاع قليل في المجموعة الأولى (24 ± 1.73 كغم²/م²) مقارنة بالمجموعة الثانية (23 ± 1.98 كغم²/م²) وهي ضمن المستويات الطبيعية.

إن leptin هو هرمون مشتق من الخلايا الدهنية يقوم بتنظيم وزن الجسم. وقد أظهرت نتائج هذه الدراسة أن مستوى هرمون leptin يظهر فرقاً واضحاً بين الرجال والنساء العراقيين، حيث أن مستوياته أعلى في النساء مما هو في الرجال. إن هذه النتائج مهمة للمساعدة في فهم الفرق بين الرجال والنساء في تنظيم تناول الطعام وزيادة الوزن وتوزيع الدهون في الجسم. إن العلاقة بين مستوى هرمون leptin ونسبة كتلة الجسم في كل من الرجال والنساء في هذه الدراسة قد يفتح باب لدراسة علاقة مستوى هذا الهرمون مع درجة خصوبة وتطور البلوغ في كل الجنسين.
INTRODUCTION

Leptin is a recently discovered hormone\(^1\) that is mainly synthesized by adipose cells and secreted into the bloodstream in amounts relative to the quantity of body fat. Plasma leptin levels in humans are strongly correlated with body mass index (BMI) and total fat mass (Figure 1) and are elevated in obese subjects.\(^2\)

Leptin is thought to have an "adipostat" function; that is, it acts as a signal informing the brain about the amount of fat stored in the body, through which the brain can regulate energy intake and energy expenditure in order to keep body weight constant (Figure 1).\(^3,4\) Therefore, when leptin levels are low, appetite will be stimulated with limited use of energy, and when leptin levels are high, appetite is reduced and energy use is stimulated.

In rodent experiments, leptin administration leads to weight loss through reduction in food intake and increased energy expenditure.\(^5\) In humans, however, leptin levels are very high in obese people, which suggests the existence of a leptin–resistance state, analogous to insulin-resistance in type 2 diabetes,\(^4\) the exact mechanisms for this resistance are not clear yet.

The leptin receptors in brain is mainly present in the hypothalamus and choroid plexus,\(^6\) where food intake is regulated through the modulation of several neurotransmitter pathways, such as neuropeptide-Y, glucagon-like peptide-1, and melanocyte-stimulating hormone pathways. Leptin may also modulate pituitary secretion of thyroid-stimulating hormone, adrenocorticotropic hormone, and gonadotropins, thus influencing indirectly the secretion of triiodothyronine / thyroxine, cortisol, and sex hormones, respectively, all of which have effects on energy balance.\(^7\)

MATERIALS and METHODS:

Forty four apparently healthy male & female (age between 23 and 35 years) were included in this study and considered as two groups to determine serum leptin concentration, BMI and fasting blood glucose for each individual.

EXCLUSION CRITERIA:

Any individual that has hyperglycemia or BMI > 25 was excluded from this study.

METHODS

Venous blood samples (6 ml) were taken from each subject to measure serum leptin and fasting blood glucose levels. The sample was transferred into a clean plain tube, left at room temperature for 30 minutes for clotting, centrifuged, and then the serum from all blood samples was separated and stored at –20 C for subsequent study.

Determination of fasting blood glucose:

Enzymatic & colorimetric method (Glucose oxidase GOD/ Peroxidase POD), the principle of this method depends on enzymatic determination of glucose according to the following reaction:\(^9\):

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{GOD}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

\[
2\text{H}_2\text{O}_2 + 4\text{ aminoantipyrine} + \text{phenol} \xrightarrow{\text{POD}} \text{quinoneimine} + 4\text{ H}_2\text{O}
\]
Determination of serum Leptin (ELISA):

Enzyme immunoassay (microtiter strips) for the quantitative determination of Leptin in human serum.  

STATISTICAL ANALYSIS:
Measurement of variables done by using numbers, percentage, means +/- standard deviation. Differences between variables were measured by using ANOVA test when it needed.

RESULT
The studied variables are listed in table 1. Serum leptin hormone levels are presented in table 2 (8.82 ± 2.9 μg/L) in group 1 & (4.65 ± 3.2 μg/L) in group 2. The variation between the two groups was statistically significant (P < 0.05). Gender differences between the two groups in serum Leptin shown clearly in Figure 2, while fig 3 shows the difference in serum leptin levels between Iraqi women and the reference values for female subjects.

Table (1) The studied variables are listed

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group –1-(n=22)female</th>
<th>Group –2-(n=22)male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.4 ± 4.18</td>
<td>30.56 ± 3.26</td>
</tr>
<tr>
<td>BMI (Kg/m²²)</td>
<td>24 ± 1.73</td>
<td>23 ± 1.98</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>116.43 ± 3.4</td>
<td>118.52 ± 2.9</td>
</tr>
</tbody>
</table>

Table (2) Serum leptin hormone levels are presented in table 2

<table>
<thead>
<tr>
<th>Variables</th>
<th>Group –1-(n=22)female</th>
<th>Group –2-(n=22)male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin hormone (μg/L)</td>
<td>8.82 ± 2.9</td>
<td>4.65 ± 3.2</td>
</tr>
<tr>
<td>Reference Value</td>
<td>7.4 ± 3.2</td>
<td>3.9 ± 2.8</td>
</tr>
</tbody>
</table>

DISCUSSION:
This study is one of the newest studies that discuss the variation in one of the newly discovered hormones (Leptin) between male & female subjects in Iraq. The knowledge of this relation is very important to understand various problems associated with obesity, puberty & infertility. From the results (table 2 & fig 2) of this study serum Leptin levels are significantly higher in women than in men (p < 0.05). This study which is done in Iraq to evaluate the values of leptin hormone in normal male & female subjects to be compared with the study of Reitman & coworkers the results shows a higher values in Iraqi female (Mean = 8.82 ug/L) than other female values (mean = 7.4 ug/L)
ug (L)³. These differences may be due to the fact that Iraqi women have higher fat mass than the other women. A comparable result was found in males with different studies. At first, such differences between Iraqi male & female subjects were thought to reflect the differences in body composition between men and women. Women in general have a higher percentage of fat mass for the same body weight or BMI. Since leptin reflects mainly the amount of body fat, this seemed to be a logical explanation for the observed gender differences. However, these gender differences and their relationship to body composition were examined, and leptin levels were found to be significantly higher in women with comparable BMI or fat mass.

A second explanation was thought in the differences in fat distribution between men and women. Women generally have more peripheral fat accumulation (especially at the level of the hips), whereas obese men have more abdominal (especially visceral) fat. It was shown in several in vitro studies that subcutaneous adipocytes produce more leptin than fat cells derived from the omental fat depot; this is especially true in women. In vivo studies also showed that leptin levels had a stronger association with peripheral or subcutaneous fat than with intra-abdominal or visceral fat, as measured by computed tomography scan. Thus, the fact that women have more subcutaneous fat, which secretes more leptin, seems an additional reason for the higher leptin levels in women. However, even after correcting for the amount of subcutaneous fat, leptin levels are still found to be significantly higher in women.

CONCLUSION

The results of this study are potentially important for the understanding of differences between men and women in regulation of food intake, weight gain, and body fat distribution; but since these differences in body composition between men and women may not be the only reason to explain the differences in leptin levels completely, other factors must play a role, like Steroid hormones but the exact mechanism or interaction is not yet known. These findings seem to indicate that this recently discovered hormone may play a role obesity, infertility & other disease states.

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Synthesis of 3’-3’- gem – di – C – Nitromethyl Nucleoside Analogues of Possible Biological Activity
Received 31-8-2002 Accepted 20-2-2005

ABSTRACT

Synthesis of new nucleoside analogues of the type : 3’, 3’- gem – di – C – nitromethyl , expected to have useful application in the chemotherapeutic treatment of AIDS, cancer and microbial infections. The synthesis involved the condensation of the appropriate sugar derivative (i.e. 3’, 3’- gem – di – C – nitromethly – 1- ribofuranose ) with nitrogen bases , such as , uracil and theophyllin following a multi step scheme starting from diacetone galucose (1) (scheme 1). The prepared compound were identified by spectroscopic methods ; ir , mass , 1H and 13C nmr.

INTRODUCTION

The 2’, 3’– didexy nucleosides have shown importance in several established chemotherapies (anticancer , antiviral and antibacterial) and other attractive field like immunomodulation or regulation of gene expression which may constitute new therapeutic approaches (13) . The board application of modified nucleosides especially in the inhibition of the human immunodeficiency virus (HIV)4,5 , have targeted the investigation for utilizing new nucleoside analogues .Efforts have primarily focused on modification of the carbohydrate portion of the natural nucleosides because cellular kinases are more tolerant of these changes than changes within the base moiety6. These observations , have led us to commit the synthesis of a variety of nucleoside analogues containing 3’, 3’- gem – di – C – nitromethyl substituent at the sugar portion .

MATERIAL and METHOD

Melting points were determined using electrothermal melting point apparatus and are uncorrected .IR spectra were recorded using either , Shimadzu (408) Jasco (J – 0085) infrared spectrophotometer . 1H – NMR and 13C – NMR spectra were determined on a Varian XR – 3005 (pharm 300) spectrometer at 299.908 and 75.4118 MHz respectively . CDC13 or DMSO was used as the solvent and TMS as internal standard . General mass spectrometer model 511 valzar was used for recording mass spectra .TLC was performed on glass plants coated with 0.25mm layer of silica gel (Fluka) and spot were detected by iodine vapour . Column chromatography was carried out with silica gel 60 ( Fluka ) . Uracil from Merck Company . 1: 2 : 5.6 – Di – O – Isopropylidene – α – D – glucofuranose (1) was prepared from 1:2 : 5.6 – Di – O – Isopropylidene – α-D – ribo – hexofuranose – 3 – ulose (2) was prepared as previously described (19) .

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*** College of pharmacy, University of Baghdad.
1.2 : 5.6 – D – O – Isopropylidene – 3 – C – nitromethyl – – D – allofuranose (3) and 3 – deoxy – 1.2 : 5.6 – di – O – isopropylidene – 3 , 3 – gem – di – C nitromethyl – – D – ribo – hexofuranose (5) were prepared as previously (8). 3 – Deoxy – 1.2 : 5.6 – di – O – isopropylidene – 3 – C – nitromethylene – – D – ribo – hexofuranose (4) was prepared according to the method described (9). Bis (theophylline – 7 – yl)mercury (10) was prepared as in the method described (11). 2,4 – Bis (trimethylisly uracil (13) was synthesized as described before (15).


3 – Deoxy – 1.2 : 5.6 – di O – isopropylidene – 3,3 – gem – di – C nitromethyl – – D – ribohexofuranose (5) (8.3g, 22.9 mmol) was dissolved in methanol and IN H2SO4 (30ml) was added. The reaction mixture was left to stand at room temperature for 3 hr. The resulting mixture was then neutralized by adding solid sodium hydrogen carbonate and then extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulphate and then evaporated. The diol (6) was purified by recrystallization from ethanol (40ml) and then stirred, saturated solution of sodium hydrogen carbonate (2ml) was added followed by a solution of sodium metaperiodate (1.32g, 6.2 mmol) in 70 ml water. The resulting mixture was stirred for 3 hr after which the excess sodium metaperiodate was destroyed by adding a few drops of ethylene glycol. The resulting aldehydo sugar was immediately reduced with sodium borohydride (0.12g). After the reaction mixture was kept with stirring for 4 hr, acetone (0.5ml) was added and the mixture was further stirred for 30 minutes. The solid residue was removed by filtration and the filtrate was extracted with methylene chloride, dried over anhydrous sodium sulphate and the solvent was removed to give (7) as a syrup (1.39g, 76.8%). IR (smear) ν3450 cm⁻¹ (OH). 1H – NMR δ 5.82 (d, 1H, H – 1 ) , 4.72 – 4.70 (q, 1H, H – 4 ), 4.66 – 4.60 (d, 1H, H – 2 ) , 4.10 (s, 4H , 2CH3NO2), 3.53 (d, 2H, H – 5 ), 3.16 (2s, 6H, 2CH3). 13C – NMR δ 104.32 (C – 1 ), 81.95 (C – 2 ), 58.74 , 58.40 (C – 3 ), 81.74 (C – 4 ), 84.19 (C – 5 ), 80.31 , 78.42 (CH3NO2). 1H – NMR δ 10.51 (O – CMe2 – O ) , 26.52 , 26.06 ( C (CH3)2).

The 3 – deoxy – 1.2 – O – isopropylidene – 3,3 – gem – di – C nitromethyl – – D ribofuranose (2) (2.4g, 8.2 mmol) was dissolved in anhydrous pyridine (6.7ml, 83 mmol) after external cooling with ice. Benzoyl chloride (0.96ml; 8.3mmol) was added dropwise. The reaction mixture was kept at room temperature for 24 hr then iced water was added. The resulting syrup was extracted with petroleum ether (b.p. 40 – 60 °C), then dried with anhydrous sodium sulphate, filtered and concentrated under reduced pressure. Traces of pyridine were removed by coevaporation with dry toluene. The benzoate derivative (8) was obtained as syrup (1.6g, 50%). IR (smear) ν (3100 – 3000 ) cm⁻¹ (aromatic C = O), (1710) cm⁻¹ (C = O), (1590) cm⁻¹, ( C – C). Mass spectrum, gave M + 396 (5 – benzoate derivative 8 ) , m/e 261 ( [ phCO2CH3 ] ) , 1H – NMR δ 7.47 (m, 5H, aromatic ) , 5.95 (d, 1H, H – 1 ), 4.76 – 4.63 (cm, H – 4 , H – 5 , H – 6 , H – 7 ), 4.58 (d, 1H, H – 2 ), 4.2 (d, 2H, CH3NO2), 3.95 (s, 2H, CH3NO2), 1.50 – 1.32 (2s, 6H, 2CH3). 13C – NMR δ 133.31, 130.05 , 129.85 , 128.52, 128.38 (aromatic ring carbons ) , 170.62(ph – CO ) , 156.05 ( C – 1 ) , 80.02 (C – 2 ), 60.49 (C – 3 ), 79.34 ( C – 4 ), 77.83 ( C – 5 ), 74.88, 74.62 (CH3NO2).

The acetylated sugar (9) (1g, 2.2 mmol) was treated with 50% (W/V) hydrogen bromide in acetic (3ml) for one hour then poured into to an ice cooled dichloromethane (50ml) , washed with iced water, and then with saturated aqueous solution of sodium bicarbonate to remove the remaining acid. After a final wash with iced water, the dichloromethane layer was dried over anhydrous sodium sulphate and the solvent was removed to give (11) as syrup (0.9g, 95%). The isolated sugar bromide (11) was used directly for the nucleoside synthesis.
Synthesis of theophylline nucleoside analogue


The theophylline mercury salt (10) (0.55 g, 0.98 mmol) was finely powdered, suspended in (150 ml) sodium - drier xylene and the solvent was partially distilled to remove traces of water azeotropically. When the temperature of mixture was raised to 137 °C, the residual suspension was allowed to cool (below 50 °C) . The acetylated sugar bromide (11) (0.9 gm, 1.96 mmol) in xylene was then added and the reaction mixture refluxed with vigorous stirring for 15 minutes. The xylene layer was washed with 20% aqueous potassium iodide to remove the remaining traces of the mercuric salt, washed with water, dried over anhydrous sodium sulphate and the solvent was removed to give after silica gel column chromatography with chloroform as an eluent the acetylated nucleoside (12) (0.57 g, 52% yield) as syrup in 80.4% yield. Oxidation of the diol (6) with sodium periodate effected the cleavage of C5 – C6 Bond and resulting intermediate aldehyde derivative was reduced immediately with sodium borohydride to give the 3 – deoxy – 1 , 2 – O – isopropylidene – 3 , 3 – di – gem – C – dinitromethyl – α – D – ribofuranose (5) which was obtained from D – glucose according to the method reported earlier by one of us (7). Selective hydrolysis of the 5 , 6 – isopropylidene group of the 3,3 – gem – di – C – nitromethyl – α – D – ribofuranose (5) with in sulphuric acid in methanol (8) gave the monoisopropylidene derivative (6) as a syrup in 80.4% yield. Oxidation of the diol (6) with sodium periodate effected the cleavage of C5 – C6 Bond and resulting intermediate aldehyde derivative was reduced immediately with sodium borohydride to give the 3 – deoxy – 1 , 2 – O – isopropylidene – 3 , 3 – gem – di – C – nitromethyl – α – D – ribofuranose (5).

Result and Discussion

1. Synthesis of the carbohydrate moiety of the nucleoside

3 – Deoxy – 1,2 :5,6 – di – O – isopropylidene – 3 , 3 – di – gem – C – dinitromethyl – α – D – ribofuranose (5) was obtained from D – glucose according to the method reported earlier by one of us (7). Selective hydrolysis of the 5,6 – isopropylidene group of the 3,3 – gem – di – C – nitromethyl – α – D – ribofuranose (5) with in sulphuric acid in methanol (8) gave the monoisopropylidene derivative (6) as a syrup in 50% yield. The final step in the synthesis of the protected sugar moiety before carrying out the coupling reaction with nucleo – bases , was the removal of 5 – O – 1 , 2 – acetal of 5 – O – benzoyl derivative (8) with 99% trifluoroacetic acid followed by acetylation. Acetylation of the 1 – and 2 – hydroxyl groups was performed with acetic anhydride in pyridine (9) which afforded 1,2 – di – O – acetyl – 5 – O – benzoxy – 3 – deoxy – 3,3 – gem – di – C – nitromethyl – α – D – ribofuranose (8) as syrup in 90% yield.

2. Synthesis of nucleoside analogues

For the synthesis of 7 – ( 2' – O – acetyl – 5' – O – benzoyl – 3 , 3' – di – C – nitromethyl – α – D – ribofuranosyl ) theophylline (12), the Koenigs – Knorr condensation method was followed (10). Thus treatment of 1,2 – di – O – acetylribofuranose derivative (9) with anhydrous hydrogen bromide in dichloromethane readily afforded 1,2 – di – O – bromide (11) which was used immediately because of its instability.
(Scheme 1).
The sugar bromide (11) was condensed with bis (theophylline = 7 – yl) mercury (10) as the activated base in anhydrous xylene under reflux which afforded the desired theophylline nucleoside analogue (12) , after silica column chromatography , as a syrup in 52 % yield. The theophylline (1,3 – dimethylxanthine ) base has been used because of its availability and due to the fact that only one of its nitrogen atom (N – 7 ) is reactive , therefore mixture of different nucleoside analogues may be avoided . The reaction of theophylline with mercuric chloride in aqueous alkali afforded the mercury derivative rather than the chloromercury one , and it was assigned that N – 7 was the predominant position of attachment of mercury group in the theophylline . It has also been demonstrated that the mercury derivative of theophylline couple with acylglycosyl halides at N – 7 (12 – 14 ) and involves direct displacement of the mercury group from nitrogen by the incoming acylglycosyl halide (11)

For the synthesis of 1 – (2’ – O – acetyl – 5’ – O – benzoyl – 3‘ – deoxy – 3’ ,3’ – gem – di – C – mitromethyl – β – D – ribofuranose ) – 4 – (trimethylsilyl ) uracil (14), the modified Hilbert – Johnson procedure using simply Friedel – Crafts catalysts like SnCl 4 was followed (15). The 1,2 – di – O – acetylribofuranose derivative (9) was coupled with the silylated uracil (13) in 1,2 – dichloroethane in the presence of anhydrous stannic chloride as Lewis acid . The reaction involved the conversion of the protected sugar (9) in to the reactive electrophilic 1,2 – acetylloxonium ion followed by the silylated uracil (13) attack to afford the protected uracil nucleoside analogue (14) with the regeneration of the catalyst . The formation of 1,2 – acetylloxonium ion determined the exclusive formation of the – β – anomer (14)(15 – 16 ), which was separated as white semisolid and characterized by its 1H NMR spectrum . Another fraction (14a) was separated on the silica gel column and identified from its 1H NMR spectrum as being the desilylated nucleoside (14a).

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Clinical Evaluation of a Formulated Econazol Nitrates as A Topical Solution
Laith H. Samein*

Received 5-9-2004 Accepted 20-2-2005

ABSTRACT
Econazole nitrate (EN) is considered as the most effective agent for the treatment of all forms of dermatomicosy caused by dermatophytes. It was formulated as a topical solution in our laboratories. This study was designed to evaluate the effectiveness of Econazol Nitrate in the prepared formula and compared with that of commercial brand, Pevaryl®. A total of 104 patient suffering from dermatomycoses were involved in this investigation. Both formula were applied to the affected skin region in the morning and evening from week to 16 weeks with light massage until complete healing effect was achieved. The data revealed that the percentage of cured patient treated with the prepared formula and reference formula of Econazol Nitrate 1% solution were 90.3% and 88.4% respectively also chronic cases could be largely cured by treatment with the prepared formula Econazol Nitrate 1% solution. The results of this clinical investigation showed that the prepared formula of Econazol nitrate 1% was effective as compared to that of the commercial brand, Pevaryl®.

INTRODUCTION
Econazole nitrate is (RS)-1-(2,4 diocloro- B (P-chorobezyl-oxyphenethyl) imidazole nitrate (1).

\[
\begin{align*}
\text{CH}_2\text{CH-O} \quad \text{CH}_2- & \quad \text{Cl}\text{Cl} \text{Cl}\text{HNO}_3 \\
\text{N} & \quad \text{N} \\
\end{align*}
\]

It is a white or almost white crystalline powder. M.p. about 164°C, with decomposition, very slightly soluble in water and ether: soluble 1 in 125 of ethanol (96%) 1 in 60 of chloroform and in 25 of methanol (1,2).

Econazole nitrate is used for the topical treatment of all forms of dermatomicosy caused by dermatophytes like tricophyton rubum, tricophyton mentagrophytes tricophyton tonsurans which cause tinea pedis, tinea cruris: and tinea corporis respectively (3). It is used for dermatomicosy caused by yeasts like candida albicans and condida guilliemordi (4).

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Econazole nitrate is available in a variety of dosage form such as skin cream (alone or in combination with triamcinolone), skin solution, skin lotion, vaginal suppositories and spray solution \(^{(3)}\).

It has been proven to be effective in the presence of mixed infection. The antibacterial effects of the preparation offer an additional advantage.

The purpose of this work is to evaluate clinically a selected formula for Econazole nitrate as topical solution prepared in our laboratory \(^{(5)}\).

**SUBJECTS and METHOD**

A total of 104 patients were involved in this investigation. They were diagnosed by Dermatologist Dr. A. Al-Swady working in medical city as having dermatomycetes caused by various dermatomycetes, blastomycetes and mould \((6, 7, 8, 9, 10)\). The patients age ranged from 17-70 year (average 63.4 years). They were randomly divided into two groups (52 patient in each) the patients in group I, were 17 female (33%) and 35 males (67%) were instructed to use the prepared formula of econazol nitrate 1% solution. Patients in group II, were 16 females (30%) and 36 males (69%) were given the commercial of econazol nitrate 1% solution, Pevaryl® Ciliag. All patients were instructed to apply econazol nitrate twice daily with light massage on to the affected skin region. The duration of treatment was ranged from one week to 16 weeks.

**RESULTS and DISCUSSION**

All patients involved in this study were evaluated clinically, in addition to mycological and microscopical examination before and after treatment with either the prepared formula or reference (Pevaryl®) of econazole nitrate 1% solution.

**CLINICAL EVALUATION**

The response to the treatment was graded as, cured, poor improvement and no effect \(^{(7)}\). The data showed that the percentage of cured, poor improvement and no effect in patients treated with econazol nitrate 1% solution (the prepared formula) were 90.3 %, 7.8 % and 109% respectively as shown in table -1.

**Table 1:** Clinical evaluation of 52 patients taking Econazole 1% solution.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>52</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cured</strong></td>
<td>47</td>
<td>90.3%</td>
</tr>
<tr>
<td><strong>Poor improvement</strong></td>
<td>4</td>
<td>7.8%</td>
</tr>
<tr>
<td><strong>No effect</strong></td>
<td>1</td>
<td>1.9%</td>
</tr>
</tbody>
</table>

The results of patient treated with Pevaryl® solution showed that 88.4% were cured, 9.7 % of poor improvement and only 1.9% with no effect as illustrated in table -2.

**Table 2:** Clinical evaluation of 52 patients taking Pevaryl® as a reference.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>52</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cured</strong></td>
<td>46</td>
<td>88.4%</td>
</tr>
<tr>
<td><strong>Poor improvement</strong></td>
<td>5</td>
<td>9.7%</td>
</tr>
<tr>
<td><strong>No effect</strong></td>
<td>1</td>
<td>1.9%</td>
</tr>
</tbody>
</table>

**MYCOLOGICAL EVALUATION**

Prior to therapy the fungus species could be demonstrated by a positive culture in 20 patients out of 52 patients treated with either the prepared formula or reference (Pevaryl®) of econazole nitrate 1% solution.

Table –3- showed that no more fungi were present in 14 patients, (70% ) treated with econazole 1% solution while 6 patients could not be checked mycologically, as there were no samples available due to healing of mycosis. Mycological examination in patient treated with (Pevaryl®) showed negative fungi in 13 patient (65%) as shown in table 4.
Table –3 Mycological evaluation of 20 patients taking Econazole 1% solution.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>20</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungus identification prior to therapy +</td>
<td>14</td>
<td>70%</td>
</tr>
<tr>
<td>After therapy -</td>
<td>Cured</td>
<td></td>
</tr>
<tr>
<td>Fungus identification Prior to therapy +</td>
<td>6</td>
<td>30%</td>
</tr>
<tr>
<td>After therapy</td>
<td>cured</td>
<td></td>
</tr>
<tr>
<td>No sample -</td>
<td>( cure)</td>
<td></td>
</tr>
</tbody>
</table>

Table –4 Mycological evaluation of 20 patients taking Pevaryl® as reference.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>20</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungus identification prior to therapy +</td>
<td>13</td>
<td>65%</td>
</tr>
<tr>
<td>After therapy -</td>
<td>Cured</td>
<td></td>
</tr>
<tr>
<td>Fungus identification Prior to therapy +</td>
<td>6</td>
<td>30%</td>
</tr>
<tr>
<td>After therapy</td>
<td>cured</td>
<td></td>
</tr>
<tr>
<td>No sample -</td>
<td>( cure)</td>
<td></td>
</tr>
<tr>
<td>Fungus identification prior to therapy+</td>
<td>1</td>
<td>5%</td>
</tr>
<tr>
<td>After therapy +</td>
<td>not cured</td>
<td></td>
</tr>
</tbody>
</table>

MICROSCOPICAL EVALUATION

The diagnosis was also confirmed by positive microscopical fungus identification prior to therapy in 25 patients treated with econazole 1% solution or Pevaryl®. The percentage treated with econazole 1% solution and Pevaryl® with a negative fungus were 80% and 76% respectively as shown in table 5 and 6. Microscopical examination could not be performed in 4 cases (16%) treated with econazole 1% and 5 cases (20%) used Pevaryl®, since no sample were available due to healing of mycosis.

Table –5 Microscopically results of 25 patients taking Econazole 1% solution.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>25</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungus identification prior to therapy +</td>
<td>cured</td>
<td>20</td>
</tr>
<tr>
<td>After therapy -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungus identification Prior to therapy +</td>
<td>4</td>
<td>16%</td>
</tr>
<tr>
<td>After therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No sample -</td>
<td>cured</td>
<td></td>
</tr>
<tr>
<td>( cure)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungus identification prior to therapy+</td>
<td>1</td>
<td>4%</td>
</tr>
<tr>
<td>After therapy +</td>
<td>not cured</td>
<td></td>
</tr>
</tbody>
</table>
Table – 6 Microscopically results of 52 patients taking Pevaryl as reference.

<table>
<thead>
<tr>
<th>Fungus identification</th>
<th>No. of patients</th>
<th>25</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>prior to therapy +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After therapy - cured</td>
<td></td>
<td>19</td>
<td>76%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fungus identification</th>
<th>Pat. No</th>
<th>Duration of disease in months</th>
<th>Duration of treatment in weeks</th>
<th>Cured</th>
<th>Not cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>prior to therapy +</td>
<td>1</td>
<td>12</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>After therapy - cured</td>
<td>2</td>
<td>12</td>
<td>11</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>No sample</td>
<td>3</td>
<td>14</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>24</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>13</td>
<td>3.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>60</td>
<td>14.5</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td>16</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>14</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12</td>
<td>9</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>12</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>30</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table – 7 Therapeutic results in 12 patients with chronic mycosis lasting for more than one year.

<table>
<thead>
<tr>
<th>Fungus identification</th>
<th>Pat. No</th>
<th>Duration of disease in months</th>
<th>Duration of treatment in weeks</th>
<th>Cured</th>
<th>Not cured</th>
</tr>
</thead>
<tbody>
<tr>
<td>prior to therapy +</td>
<td>12</td>
<td>1-5 years</td>
<td>2-16 weeks</td>
<td>11 pat. 95%</td>
<td>1 pat. 8.3%</td>
</tr>
</tbody>
</table>

Therapeutic Results In Chronic Cases:

12 patients of 52 patients who suffered from chronic mycosis (duration 1-5 years) were treated with the formula of econazole. The data showed that 11 patients (91.7%) were cured after treatment with an average duration of (2-16) weeks. Only one patient (8.3%) did not respond as shown in Table 7.

The appearance of any side effect after the treatment with both formulas was also monitored. Burning and pruritus (11,12,13,14,15) were observed in only 3 patients treated with econazole 1% solution (out of 52 patients).

CONCLUSION

The results of this clinical investigation clearly indicated that the prepared of econazole nitrate 1% was effective as compared to that of commercial brand Pevaryl®.
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The Role of Oxidative Stress In Lead Poisoning

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ABSTRACT

To investigate the relationship between increased lipid peroxidation, and the lowering of both plasma total proteins and albumin in lead-exposed workers, and the effectiveness of antioxidants (vit. C and E) in modulating oxidative stress in those workers.

Thirty male and females workers employed in the Iraqi storage battery (age range 20-40 years) were participating in this study. Additionally, 11 healthy subjects were served as healthy controls, with the same age range compared to workers group, to avoid the effects of age variations on the studied parameters. Blood lead levels, erythrocyte and plasma MDA, erythrocytes and plasma GSH, total protein and albumin levels in healthy controls and lead-exposed workers pre- and post-treatments with antioxidant were measured.

Comparison with healthy control groups reveal 360% increase in blood lead levels, 150% increase in erythrocyte MDA, 117% increase in plasma MDA, 28% decrease in erythrocyte GSH, 56% decrease in plasma GSH, 13% decrease in total plasma protein and 23% decrease in albumin levels in lead-exposed workers. Treatment with a combination of antioxidant vitamins (1000 mg/day vit. C and 200 mg/day vit. E) for one month produced significant reduction 12% in lead levels, 54% in erythrocyte MDA, 53% in plasma MDA; significant increase 41% in erythrocyte GSH, 120% in plasma GSH and 11% in plasma albumin levels in comparison with pre-treatment levels.

In conclusion, there is a beneficial effect of antioxidants on the oxidative stress parameters that not only related to their ability to remove lead from target cells, but also associated with antioxidant potential for bolstering thiol antioxidant capacity, and this makes these vitamins a good candidate for therapeutic intervention in lead poisoning.

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** Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad
INTRODUCTION

Lead, similar to many heavy metals, is a complex toxin, exerting numerous pathophysiologic effects in many organ systems \(^1\). At the molecular levels, lead interacts with biomolecules and functions in different ways, like binding to numerous structural and enzymatic proteins \(^2\), interference with metabolic pathways of mitochondria \(^3\), and exhibiting mutagenic and carcinogenic effects in mammalian cells \(^4\).

Oxidative stress which refers to a cellular situation characterized by elevation of the study state concentrations of reactive oxygen species (ROS), and this could be a possible contributor to the pathogenesis of lead poisoning \(^5\). Some in-vitro and in-vivo studies showed an elevated production of ROS due to lead treatment \(^6,7,8\), and increased lipid peroxidation associated with altered antioxidant defense systems \(^9\).

The effects of lead on the oxidative stress parameters like glutathione (GSH), and malondialdehyde (MDA), suggests ROS as a possible contributor to cell damage due to lead exposure \(^10\). The increase in lipid peroxidation during lead poisoning were found to be accompanied by alterations in the antioxidant defense system, including decreased GSH levels in all body compartments \(^11\).

This study was conducted to investigate the extent to which ROS-related processes are involved in lead poisoning and the possibility of therapeutic intervention with antioxidant vitamins in this case.

SUBJECT and METHOD :

This study was carried out on workers employed in the Iraqi storage battery plant, and selection was made on the basis that they must be directly involved with lead exposure, and have been employed for at least 8 months, before the investigation were carried out.

Thirty, male and females volunteers (age range 20-40 years) from the workers of the battery plant, participate in this study. Eleven healthy subjects served as healthy controls, with the same age range compared to workers group, to avoid the effects of age variations on the studied parameters.

The average working time (hrs/day) for each worker is 6 hrs, with a period of exposure to lead ranging from 8 months to 28 years.

Individual symptom survey was performed by clinical and physical evaluation of the workers involved in the study, concerning the presence of lead-associated signs and symptoms for the purpose of proper selection.

Blood samples (10 ml) were drawn by vein puncture from each subject prior to starting treatment with antioxidants (as baseline sample). After that, all subjects receive a combination of antioxidant vitamins (ascorbic acid 1000 mg/day and α-tocopherol 200 mg/day) orally for a period of 4 weeks, then second blood sample was drawn for evaluation of the effect of treatment on the studied parameters.

Blood samples were placed into heparinized tubes and refrigerated until separation of erythrocytes and analysis.

Blood lead levels were measured by graphite France atomic absorption spectrophotometer according to the method of Parson et al \(^12\).

Erythrocytes and plasma malondialdehyde (MDA) level as indicator of lipid peroxidation were assessed utilizing thiobarbituric acid assay method of Stock and Dormandy \(^13\), and the susceptibility of plasma and erythrocytes to in-vitro hydrogen peroxide-induced oxidative stress was measured according to the method of Gilbert et al \(^14\), and the results were expressed as nmole (MDA)/gm Hb, based on the molar extinction coefficient of MDA (1.56x10^5 M^-1 cm^-1). Glutathione levels were determined in erythrocytes and plasma according to the method of Godin et al \(^15\). Plasma albumin levels were determined utilizing a ready-made kit for this purpose (Randox company, England) according to the method of Doumas et al \(^16\). Total plasma protein was measured according to the Biuret method \(^17\). Erythrocyte hemoglobin concentration was measured using Drapkin’s reagent method \(^18\).

Statistical analysis of the data was done using Student’s t-test, and P-values of less than 0.05 were considered significant.

Table (1) showed a significant elevation in lead levels in blood of exposed workers (360%) compared to controls, produced 12% decrease in lead levels, which is a significant value compared with pre-treatment levels.

The results of the study indicates that the base line erythrocytes and plasma MDA levels were elevated by 150% and 117% respectively compared to that of controls (Table 1). MDA levels in both compartment decrease after treatment with a combination of antioxidant vitamins (1000 mg/day ascorbic acid and 200 mg/day (α-tocopherol) for one month, which was significant, compared to the pretreatment values (Table 1). The response of erythrocytes and plasma to in-vitro hydrogen peroxide challenge showed that, MDA production in both compartments of the exposed worker’s blood were significantly higher, compared to that of controls. Treatment with antioxidants as indicated before, significantly increase the
resistance of erythrocytes and plasma of lead-exposed subjects to the hydrogen peroxide-induced lipid peroxidation, reflected by a significant decrease in the MDA production after antioxidant treatment, compared to pretreatment levels (Figures I and II).

Table (1): Blood lead levels, erythrocytes and plasma MDA levels in lead-exposed workers pre- and post-treatments with vit. C and vit. E

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control n = 11</th>
<th>Lead exposure workers Before treatment n = 30</th>
<th>After treatment n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood lead μg/dl</td>
<td>10 ± 0.64</td>
<td>46.4 ± 0.07*</td>
<td>41.03 ± 1.96*</td>
</tr>
<tr>
<td>Erythrocyte MDA nmole/gm Hb</td>
<td>7.7 ± 0.29</td>
<td>19.81 ± 20*</td>
<td>9.07 ± 0.58*</td>
</tr>
<tr>
<td>Plasma MDA nmole/L</td>
<td>0.97 ± 0.08</td>
<td>2.11 ± 0.08*</td>
<td>1.00 ± 0.06*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SE

* Significantly different from control (P<0.05).

¥ Significantly different with respect to pre-treatment (P<0.05).

In lead-exposed workers, there was 28% and 56% depletion in erythrocytes and plasma glutathione (GSH) levels respectively, observed before antioxidant treatment, and compared to controls (Table 2). After treatment with antioxidants for one month, there were a significant increase in GSH levels in both compartments (41% and 120% respectively) compared to pretreatment levels (Table 1). Total plasma protein and albumin, the general antioxidants in the body, were found to be affected due to lead exposure, and their levels in lead workers were significantly decreased (13% and 23% respectively) compared to controls. Antioxidant treatment produced a significant elevation on plasma albumin only after one-month duration of treatment (Table 3).
Table (2): Erythrocytes and plasma glutathione (GSH) levels in lead-exposed workers pre- and post-treatment with vit. C and vit. E.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control n=11</th>
<th>Lead exposure workers Before treatment n=30</th>
<th>After treatment n=30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte GSH</td>
<td>11.92 ± 0.36</td>
<td>8.62 ± 0.4*</td>
<td>12.17 ± 0.37* v</td>
</tr>
<tr>
<td>μmol/gm Hb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma GSH</td>
<td>0.88 ± 0.15</td>
<td>0.39 ± 0.05*</td>
<td>0.86 ± 0.12*</td>
</tr>
<tr>
<td>μmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean ± SE
* Significantly different from control (P<0.05).

Table (3): Plasma total protein and albumin levels in lead-exposed workers pre- and post-treatment with vit. C and vit. E.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control n=11</th>
<th>Lead exposure workers Before treatment</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma protein gm/dl</td>
<td>7.51 ± 0.11</td>
<td>6.56 ± 0.13*</td>
<td>6.6 ± 0.12*</td>
</tr>
<tr>
<td>Plasma albumin gm/dl</td>
<td>5.06 ± 0.1</td>
<td>3.88 ± 0.05*</td>
<td>4.29 ± 0.11*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SE
* Significantly different from control (P<0.05).

DISCUSSION

Free radicals activity has been implicated in the pathogenesis of a variety of human diseases and the analysis of our data showed that, oxidative stress was quite clear in lead exposed workers (Table 1, Figure I and II), as noticed by increased erythrocytes and plasma MDA levels, which is in agreement with other studies (19, 20).

The mechanisms by which lead causes it’s deleterious effects has yet to be elucidated, however, part of lead’s effect may be due to the accumulation of delta-aminolevulinic acid dehydratase (ALAD), an enzyme in the heme synthesis pathway, which catalyzes the condensation of two molecules of δ-ALA to porphobilinogen (21). At a pH range of 7.0-8.0 δ-ALA enolizes, and the resulted enol undergo autooxidation resulting in the formation of superoxide and hydroxyl radicals. ALA has also been shown to undergoes iron-catalyzed oxidation with ROS generation, and to induce Ca²⁺ release from mitochondria through oxidative damage to inner membrane (22).

The effect of antioxidant vitamins (ascorbic acid and tocopherol) on lipid peroxidation parameter, MDA, as shown in table (1) and figure I and II, suggested that they did produce a decrease in the basal MDA levels and the susceptibility of both, erythrocytes and plasma to the oxidative stress induced in-vitro by H2O2. The antioxidant treatment lead to increase in erythrocytes and plasma GSH levels (Table 2), which may be due to a direct scavenging activities of the generated ROS, and decreasing utilization and damage of GSH, or indirect through the improvement of the oxidant/antioxidant balance in the cells after treatment (23).

In normal conditions, as well as during oxidative stress (lead exposure) a daily dose of ascorbic acid and α-tocopherol, appear to be protect the oxido-reductive state of red blood cells, by modulating the extent of lipid peroxidation, as well as the activities of the antioxidant enzymes (24).

In this study, daily supplementation with a combination of ascorbic acid and α-tocopherol, resulted in a significant decrease in lead levels in the blood after one month (Table 1), and this may provide an economic and convenient method of reducing blood lead levels, possibly by decreasing intestinal absorption of lead (25), or it may increase the renal excretion of this metal.

Albumin is known to act as an effective antioxidant, due to its ability to bind the catalytic copper ions (26), free fatty acid, and hypochlorous acid (HCl), and also showed a significant capability to destroy H2O2 in the presence of reduced glutathione (27).

The present study clearly demonstrated the relationship between increased lipid peroxidation, and the lowering of both plasma total proteins and albumin in lead-exposed workers (Table 3), which may be attributed to the structural modification, which may lead eventually to impair the antioxidant properties of albumin, and even may act to induce oxidative stress, through its action as a prooxidant in presence of catalytic ions (28).
Antioxidants treatment resulted in significant elevation in albumin levels (Table 3), which may be due to their direct scavenging activity, or protection of albumin against ROS-induced damage.

In conclusion, results of this study suggested that the beneficial effects of antioxidants on the oxidative stress parameters are not only related to their ability to remove lead from target cells, but also associated with antioxidant potential for bolstering thiol antioxidant capacity, and this makes these vitamins a good candidate for therapeutic intervention in lead poisoning.

**REFERENCE**


Conjugation of Steroidal and Non – Steroidal Anti-Inflammatory Drugs as Possible Mutual Prodrug

Muthanna D. Saud*

Received 30-12-2002 Accepted 15-5-2005

ABSTRACT

Prednisolone (SAID) was conjugated with ibuprofen (NSAID) through an amino acid (glycine) as a spacer arm to synthesize the following compound:

Prednisolone – glycine – ibuprofen.

The method employed consists of converting the carboxylic acid function of (R,S) – ibuprofen – glycine to the highly reactive acid chloride and subsequent reaction with the C21 hydroxyl group of prednisolone. This reactive intermediate was found to react as well with the C17 tertiary hydroxyl group of the steroid to form three compounds and eight diastereomers. These results were confirmed by T.L.C, and the desired compound was separated by column chromatography. The identity of the prepared compound was established using U.V spectroscopy, IR spectroscopy and elemental microanalysis. The partition coefficient (PC) for this compound was estimated and found to be more soluble in the organic phase (n-octanol). Preliminary kinetic study indicated that the compound needs more than 15 hours for significant hydrolysis in phosphate buffer pH 7.8.

INTRODUCTION

Drug targeting to specific receptors or specific organs has been one of the main objectives of the medicinal and pharmaceutical chemists from the beginning of the past century. However, only in the past 30 years or so have there been any promising developments in achieving this goal13. The site – specific delivery of drug is indeed a very attractive goal because this provides one of the most significant potential ways to improve the therapeutic index of the drugs6. When a drug is delivered preferentially to the site of the action by virtue of this desired differential distribution, it will spare the rest of the body; thus it will be significantly reduce the overall toxicity while maintaining its therapeutic benefits3.

One of the approaches for site – specific drug delivery is the chemical approach or so called site – specific chemical delivery systems (CDSs) which provide a wide variety of possibilities for site – enhanced or site – specific delivery14, which are reactions by which the parent drug is covalently coupled with one or more carrier moieties. By design, after delivery the CDS will undergo a variety of enzymatic conversions, which produce intermediates all having different physical properties and varying rates of formation and elimination, thus ultimately allowing a preferential and favorable distribution of the precursor prodrug at the site of the action where ultimately the drug is released5,6. Colon – specific delivery of bioactive compounds received extensive investigations, utilizing the significantly variable bioenvironments of the different parts of the G.I.T.8,9.

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Corticosteroids were currently used for the treatment of inflammatory bowel diseases. They are used either alone or in combination with other drugs. In a recent investigation in this laboratory dexamethasone (SAID) was conjugated to metronidazole through a phosphodiester linkage. This possible prodrug, which was found to be insoluble in the aqueous medium at low pH, was suggested to be able to reach the lower part of the G.I.T. in which it will gain enough aqueous solubility to be hydrolyzed through enzymatic and/or non-enzymatic processes to liberate its active moieties. In this investigation we would like to report the synthesis of the following conjugate:

**Prednisolone – glycine – ibuprofen**

Ibuprofen – glycine conjugate, which was previously synthesized, was converted to the acid chloride through reaction with thionyl chloride. This reactive intermediate was allowed to react with the C21 hydroxyl group of prednisolone to form the final conjugate. The reactive intermediate was found to react, though to a much lesser extent, with C17 hydroxyl group of the cortisone as will be described in the following sections.

**EXPERIMENTAL SECTION**

**Materials:**

The amino acid glycine was purchased from HOPKINS and WILLIAMS LTD, England. Prednisolone and ibuprofen were a gift from the Jordanian Pharmaceutical Manufacturing Company LTD. The identity and purity of these compounds were checked according to the B.P and Merck Index. N,N’-dicyclohexylcarbodiimide (DCC) was from ACROS USA. The remaining chemicals were of reagent grade, and were used as such without further purification, since they were of the highest commercially available purity.

**General Methods:**

All reactions, throughout this work that need a constant temperature, were carried out in a thermostated double jacketed flask connected to a constant temperature circulator and refrigerator of Ultra – temp 2000 Jullablo VC. Chiller, Germany. Melting points were measured using an electrothermal melting point apparatus and were uncorrected. Thin layer chromatography (TLC) using silica gel coated glass plates was performed to follow up chemical reactions. The purity of the prepared compounds was checked by thin layer chromatography plates of (20X20) silica gel (60 F254) with 0.25mm layer thickness obtained from merck, Germany. Chromatograms were eluted by one of the following solvent systems:

A: Menthol: Ammonia (100:1.5 V:V)
B: Benzene: Ether: Acetic acid: Menthol (120:60:18:1 V:V)
C: Chloroform
D: Acetone: n – Hexane (33:67 V:V)
E: Benzene: Ether: Menthol (60:35:5 V:V)

The chromatographic spots were revealed by either reactivity with iodine vapor or by observing them under UV light. IR spectra were recorded on Perkin – Elmer spectroscopy, England. UV spectra were carried out at the National Center for Pharmaceutical Research and Quality Control, Baghdad, using Cecil L – 411, France. Column Chromatography were carried out using glass column (75cmX20mm) prepackaged with 50gm of silica gel (Kieselgel 60) suspended in 100ml of chloroform. Elemental Micro Analysis (CHN) was performed at the University of Mousel, College of Science using (CHN) analyzer type 1106 Carlo Erba. The pH values were measured using Pye Unicam pH meter (Philips), Holland.

**Chemical Synthesis:**


Ibuprofen – glycine (1.5gm, 5.7m mol) which was previously synthesized, was dissolved in 10ml chloroform and the solution was cooled to 5C. An excess thionyl chloride (2.5ml) was added drop wise with continuous stirring, during which the temperature of the reaction mixture was kept below 10C. The mixture was then refluxed for more than 2hrs. until the evolution of gaseous SO2 and HCl were ceased. The solvent was evaporated to dryness in vacuo and the residue was redissolved in chloroform and evaporated. This process was repeated several times in order to remove excess thionyl chloride. Ibuprofen – glycine acid chloride was obtained as a faint yellow oily residue and was used as such for reaction with prednisolone.

Ibuprofen – glycine acid chloride (1.4gm, 5mmol) was dissolved in 10ml of dry dichloromethane, and the solution was cooled to 0ºC. Prednisolone (2.52gm, 7mmole) was dissolved in 200ml acetone and 1.5ml of triethylamine was added. The cortisone solution was added drop wise to the cooled acid chloride solution over a period of 3 – 4hrs with continuous stirring. The reaction mixture was then stirred at 25ºC for 48hrs.

After that it was filtered to remove triethyl ammonium chloride and the filtrate was evaporated to dryness under vacuo to a very thick brown past.

The pasty residue was dissolved in ethyl acetate and washed with 0.1N HCl, then with distilled water, then with 5% NaHCO₃ solution, twice with distilled water. The ethyl acetate layer was dried over anhydrous calcium chloride and filtered.

Compound II was then separated as diastereomeric mixture by column chromatography using solvent system E as the mobile phase. Many attempts were performed to crystallize the past product but all were failed.


Elemental analysis, calculated for C₃₆ H₄₇ NO 7 – H₂O: C; 69.34, H; 07.86, N; 2.24, found: C;70.12,H;07,52,N;2.55.T.L.C; Rf values; 0.83(A); 0.35 and 0.4 (B); 0,18(C); 0.75(D); 0.52 and0.61(E).

Fig(1) UV Spectrum of Compound II

Determination of Partition Coefficient:-

Partition coefficient (PC) for a solute could be determined using the following relation:

\[
PC = \frac{C_o}{C_w}
\]

Where Co = the concentration of the solute in organic phase, and Cw = the concentration of the solute in the aqueous phase.

Partition coefficient for compound II has been performed by adding 25mg of the solute to a separatory funnel containing 25ml of water pre – saturated with octanol and 25ml of octanol pre saturated with water. The separatory funnel was inverted several times during 30min, after that it was left for complete separation of the two phases. The aqueous phase was analyzed for the solute.

A standard curve had been constructed by measuring the absorbance of different concentrations of compound II, (figure 2). The
partition coefficient for our compound was found to be 82.3.

On the other hand the stability of compound II in phosphate buffer (0.1M, pH 7.8) was determined over a period of 24hrs by incubation of 100mg of compound II in 10ml of aqueous phosphate buffer at 37ºC. An aliquot (2ml) of each sample was taken at certain intervals (30min, 10hrs, 20hrs, and 25hrs) and was measured for the remaining amount of compound II by converting the absorbance to the corresponding concentration. A plot was constructed, concentration of the remaining amount of compound II versus time (figure 3).

**RESULT and DISCUSSION**

**Synthesis of Ibuprofen – Glycine – Prednisolone (Compound II)**

This was performed by reaction of ibuprofen – glycine acid chloride with the C21 hydroxyl group of prednisolone in the presence of triethylamine to abstract the liberated HCl. Prednisolone (scheme 1) has three hydroxyl groups, a primary hydroxyl at C21, a secondary hydroxyl group at C11 and a tertiary one at C17. Because of the high reactivity of acid chloride, reaction with C17 hydroxyl group could also be occurred. On the other hand reaction with the C11 hydroxyl group had been excluded because C11 hydroxyl group is sterically hindered due to the presence of two methyl groups at C10 and C13.

Accordingly, compound II is not the only one that is formed through esterification, compound III is also formed, though to a lesser extend, by esterification with C17 hydroxyl group. In addition to that esterification of both C21 and C17 was found to be occurred to form compound IV (scheme 1).

Rearrangement of the C17 ester (compound III) to the more stable C21 ester might takes place in the presence of aqueous or non – aqueous medium. This rearrangement is faster than hydrolysis to the parent steroid.

**Stereochemistry:**

Ibuprofen used in this investigation contains one chiral center and exist as a racemic mixture composed of equal amounts of two enantiomers having R and S configurations. Accordingly, ibuprofen –
glycine acid chloride (compound I, scheme I) will exist in these two configurations. Prednisolone, on the other hand, is an optically active molecule which has more than one chiral center. Because the reaction we are dealing with does not affect any chiral center at prednisolone molecule, the configurations of these chiral centers remain constant. As a result, the specific rotation of prednisolone will not be changed and for simplification we arbitrary considered it to has an S configuration. Based on these considerations, it was expected that the major product (compound II, scheme I) will exist in two diastereomeric forms, (S,S), and (S,R). On the other hand, when esterification occurs at C_{17} hydroxyl group of prednisolone (compound III), it will also generate two diastereomers. Finally, when both C_{17} and C_{21} hydroxyl groups of prednisolone have been esterified (compound IV), this situation will result in the formation of four diastereomers (S,S,S), (S,S,R), (S,R,S), and (S,R,R). Accordingly, there will be eight diastereomers resulted from this reaction. In a previous work, when dicyclohexylcarbodiimide had been used for conjugation of hydrocortisone with ibuprofen, esterification was found to occur exclusively at C_{21} hydroxyl group of hydrocortisone\(^{(13,18)}\).

**Separation of Diastereomers:**

Of a number of T.L.C. solvent systems experienced in this investigation, system E was found to give the best separation of diastereomers. This solvent system had been used successfully by other investigators for the separation of different diastereomers\(^{(15)}\). Using this system, the final product, after purification, showed eight spots of significantly different Rf values: 0.37, 0.44, 0.52, 0.61, 0.71, 0.77, 0.85 and 0.91. Because of the low polarity of this solvent system, the relatively non-polar diastereomers of compound IV will expected to move faster and be in the upper part of the plate. The diastereomers of compound III being having the highest polarity in comparison with the other two compounds, will move the shorter distances and appear at the lower part of the plate. Compound II diastereomers which have relatively medium polarity will expected to occupy the middle situation on the T.L.C. plate. These rational expectations had been the basis for separation of the diastereomers of compound II through column chromatography which were further confirmed by IR spectroscopy and C.H.N analysis. Compound II that was synthesized and identified throughout this work, was found to has relatively high partition coefficient which gave an indication of low solubility in the aqueous gastric fluid\(^{(20)}\). Moreover it has relatively high molecular weight a reason that will decrease the possibility of its absorption through G.I.T. A preliminary investigation of its stability at aqueous phosphate buffer, pH 7.8, indicated that its significant hydrolysis took about 15 – 25 hours, during which it will expected to reach the lower parts of G.I.T where it may liberate its active species therein.
REFERENCES
Phosphodiester Conjugation of Metronidazole and Dexamethasone as Possible Mutual Prodrug
Muthana D. Saud* and Suhair M. Ghani
Received 30-12-2002 Accepted 15-5-2005

ABSTRACT
As possible mutual prodrug had been synthesized that contain metronidazole and dexamethasone conjugated through phosphodiester linkage. The rationale for this type of conjugate is to get a prodrug with possible site – specific delivery of its active constituents into the lower parts of the G.I.T. This compound was synthesized by the reaction of dexamethasone – 21 – phosphate with metronidazole to form: (1 – (dexamethasone – 21 – phosphoryl) – metronidazole)
This conjugate was performed using dicyclohexylcarbodiimide (DCC) as a condensing agent. The identity of the prepared compound had been confirmed using T.L.C., U.V. spectroscopy, IR spectroscopy and elemental analysis.
The partition coefficient for it had also been determined through n – Octanol / water partitioning system.

INTRODUCTION
Metronidazole (2 – Methyl – 5 – nitroimidazole – 1 – ethanol). (Flagyl, others), is a synthetic antimicrobial agent which was found to have particularly high activity in vitro and in vivo against a wide variety of anaerobic protozoal parasites and anaerobic bacteria\(^1,2\). Recent studies indicated that a combination of metronidazole and an anti- inflammatory steroid is one of the most effective regimen for the treatment of inflammatory bowel disease including ulcerative colitis and cohn's disease\(^3\).
In order to optimize drug action new drug formulation have been developed based on advanced technological delivery system\(^4,5\) or the pro – drugs approach\(^6,7\). Pro – drugs should be seen in the light of the still growing need for delivery system which may enable one to transport active agent selectively to the target and consequently release the drug over desired period of time.

Several types of ester pro – drugs for metronidazole had been synthesized utilizing pro – moieties that impart different physicochemical properties for this drug through the hydroxyl functional group of it. Most of these pro – drugs were synthesized in an attempt to increase water solubility of metronidazole to be used in preparing parental dosage forms\(^8,9,10,11\).
On the other hand a wide variety of corticosteroids pro – drugs have been synthesized and are in clinical use. These pro – drugs were synthesized utilizing the C – 21 hydroxyl group to make esters of different physicochemical properties\(^12\).
Corticosteroids were used in these pro – drugs either as promoiety for targeting the active species as in the case of anticancer agents\(^13,14\), or as the active species which were conjugated to promoieties to change

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the physicochemical properties\textsuperscript{(12)}, or for targeting these corticosteroids to the desired tissue\textsuperscript{(15,16)}.

Mutual pro-drugs for corticosteroids were also synthesized through conjugation with non-steroidal anti-inflammatory drugs using an amino acid as spacer arm\textsuperscript{(17)}.

The present report describes the synthesis of a compound containing dexamethasone and metronidazole linked through a phosphodiester linkage. This possible mutual pro-drug was designed, through its inherent physicochemical properties to be cleaved in the lower part of the GIT especially the colon.

**EXPERIMENTAL SECTION**

**Materials:**

Metronidazole, was a gift from Samarra drug industry, dexamethasone sodium phosphate was a gift from Jordanian pharmaceutical manufacturing company LTD. The purity of these two compounds was checked according to the B.P and Merck Index.

N,N-dicyclohexylcarbodiimide (DCC) was purchased from ACROS, USA. The remaining chemicals were of reagent grade, and were used as such without further purification, since they were of the highest commercially available purity.

**General Methods**

All reactions, throughout this work that need a constant temperature, were carried out in a thermostated double jacketed flask connected to a constant temperature circulator and refrigerator of ultratemp – 2000, Jullabo VC.

Melting points were measured using an electrothermal melting point apparatus and are uncorrected. Thin-layer chromatography (T.L.C) using silica gel coated glass plates was performed to follow up chemical reaction. Purity of the prepared compound was checked by thin-layer chromatography plates 20x20cm of silica gel 60 F\textsubscript{254} with 0.25mm layer thickness, Merck, Germany. Chromatograms were eluted by the following solvent systems:


The chromatographic spots were revealed by either reactivity with iodine vapor or by observing them under UV light.

UV spectra were recorded on Pye Unicam UV spectrophotometer, SP 8 – 0100 Germany. IR spectra were recorded on Pye Unicam SP – 300 spectrophotometer, Germany.

C – H – N analysis was performed at the University of Mosul, College of Science, Using (C.H.N) analyzer, type 1106 Carlo Erba.

**Chemistry:**

**Synthesis of 1 – (Dexamethasone 21-phosphoryl) – Metronidazole, Compound I, Figure (1).**

![Fig.1 chemical structure of compound I](image)

**General Procedure\textsuperscript{(18)}:**

Dexamethasone phosphate, sodium salt, 6.169g (12mmol) was dissolved in the minimum volume of distilled water. To this solution was added dilute HCl solution drop wise with stirring until the complete precipitation of the free phosphoric acid ester of dexamethasone. The suspension was filtered, and the white precipitate of the free acid was collected, washed with distilled water and dried.

The free phosphoric acid ester of dexamethasone was dissolved in 150m of anhydrous pyridine. To this solution was added 4.9g (24 mmole) of (DCC) and the mixture was stirred at 25ºC for 2hrs, during which dicyclohexylurea (DCU) was precipitated and filtered. To the filtrate there was added 1.026g (6mmole) of metronidazole and the reaction mixture was stirred for two days at 25ºC. The mixture was evaporated to dryness, and then the final traces of pyridine were removed by coevaporation with toluene (50ml).

The residue was treated with 200ml of 50% aqueous ethanol, and the insoluble DCU was removed by filtration. The filtrate was evaporated to dryness, redissolved in 100ml of 50% aqueous ethanol, and applied to a silica gel column (3.5x26cm) prepacked with 50% aqueous ethanol. The material was then eluted...
using isopropyl alcohol – water – concentrated ammonium hydroxide (7:2:1) as the mobile phase. The fractions between 65 and 125ml were pooled, evaporated to dryness. A yellowish to white crystalline powder was collected as the ammonium salt of compound 1; M.P 128 – 130ºC, the yield was 45%. The UV spectrum of the compound (0.1mg/ml distilled water) show λmax at 320nm, λmax for dexamethasone phosphate and metronidazole were found to be 243nm and 264nm respectively (Figure (2)).

**Fig.2 U.V spectrum**

D = Dexamethason sodium phosphate  
M = Metronidazol 
I = Compound 1, Solvent : Distilled water

IR spectrum (Figure (3)) revealed the following absorption frequencies, cm⁻¹, (Nujol): 3232 (O–H) Hydrogen bonded; 2939 (C–H aliphatic); 1720 (C=O); 1662, 1612 (C=O, C=C, C=N); 1490, 1242 (P=O); 1535 (-NO₂ group); 1072 (P – O – C); 887 (C – N) stretching for hetero aromatic compound.

**Fig.3 IR Spectrum of Compound 1**

T.L.C for compound [I], Rf value, solvent system, A (0.88), solvent B (0.53). Table (1). Elemental analysis calculated for C₂₈H₃₇O₁₀N₃PF., NH₄H₂O C, 50.83; H, 6.50; N, 8.47 found C, 51.26; H, 6.93; N, 8.13.

**Table 1: The Rₐ values for the reactants and compound (I) using two different solvent systems, where:Solvent**

<table>
<thead>
<tr>
<th>The substance</th>
<th>Solvent System</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound (I)</td>
<td>A</td>
<td>0.88</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>A</td>
<td>0.96</td>
</tr>
<tr>
<td>Dexamethasone phosphate</td>
<td>A</td>
<td>0.72</td>
</tr>
<tr>
<td>Compound (I)</td>
<td>B</td>
<td>0.53</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>B</td>
<td>0.7</td>
</tr>
<tr>
<td>Dexamethasone phosphate</td>
<td>B</td>
<td>0.35</td>
</tr>
</tbody>
</table>

A: isopropyl alcohol – water conc.  
Ammonium hydroxide (7:2:1).  

**Determination of Partition Coefficient**

The partition coefficient (PC) of a solute is defined as the ratio of the concentrations of solute distributed between two immiscible solvents at equilibrium, and it is usual to present the ratios that in favour of organic phase:

\[
PC = \frac{C_0}{C_w} \quad \text{-- (I)}
\]

Where Co = the concentration of the solute in the organic phase, and  
Cw = the concentration of the solute in the aqueous phase.

Partition coefficient for compound [I] had been performed by adding 50mg of the solute to a separatory funnel containing 50ml of water per – saturated with octanol and 50ml of octanol per – saturated with water. The separatory funnel was inverted several times during 30min after that separatory funnel was left for complete separation of the two phases.  
The aqueous phase was analyzed for the solute.  
A standard curve had been constructed by measuring the absorbance of different concentrations of compound [I], Figure (4).  
The partition coefficient for our compound was calculated according to equation (I), and was found to be 0.42.
RESULTS and DISCUSSION

Compound [I] had been synthesized through the following steps:

In the first step dexamethasone phosphate disodium salt was converted to dexamethasone phosphate acid ester by acidification with dilute hydrochloric acid (Equation I) (Scheme I).

In the second step, dexamethasone phosphoric acid ester was converted to the phosphate anhydride using dicyclohexylcarbodiimide (DCC) as a dehydrative coupling reagent, Eq. (2).

The reactive anhydride thus formed was allowed to react with the primary hydroxyl group of metronidazole which acts as a nucleophile that attack the phosphate.

The method used for the synthesis of compound [I] is a modification of procedures well established in the area of nucleotide synthesis(19,20), as well as for conjugation of nucleosides with corticosteriodes through phosphate diester linkage (21).

The coupling agent DCC, was introduced by Khorana during nucleoside polyphosphate synthesis to promote synthetic reactions involving dehydration(22).

This reagent was then used by Sheehan et al., as a condensing agent for amide bond formation during peptide synthesis (22).

Pyridine, during this course of reactions, has two roles. It acts as a powerful solvent and as a catalyst according to the following (Scheme II) (24).

DCC was used successfully in this laboratory as a condensing agent for many reactions that involve synthesis of carboxylic acid esters, and for amide bond formation as well (23).

In a previous work (12), attempts at condensation of prednisolone 21 – phosphate or prednisone 21 – phosphate with 5 – hydroxyl group of nucleosides in the presence of DCC and pyridine at room temperature and at reflux were not successful. This failure could be attributed in part as due to the method applied in which the prednisolone 21 – phosphate was
not converted to the free acid ester prior to reaction with DCC and pyridine. In addition to that, the procedure employed did not allowed sufficient time for the phosphate anhydride formation prior to the reaction with the alcohol functionality, as we did in our procedure. Confirmation of the molecular structure of compound [I] was provided by elemental analysis, UV, IR and TLC. Lipophilicity is a term commonly used to describe the tendency for a chemical agent to partition itself between aqueous and organic biophases. Partition coefficients provide a convenient measure of lipophilicity and are often used in establishing the relative rates with which chemical substances penetrate lipoidal membranes or participate in the formation of a hydrophobic bond\(^\text{26}\).

Partition coefficient as was previously defined, is an important parameter for measuring the relative affinities of the solute for an aqueous and non-aqueous or lipid phase. The greater the value of \( PC \) (equation (1)), the higher the lipid solubility of the solute. On the other hand, the lower the value of \( PC \) the higher the aqueous solubility for the solute.

\( n \)-Octanole is the solvent for which most partition coefficient values have been published. It is claimed that the octanol/water system is satisfactory model for the biological system because the organic phase is not completely non polar and contains a significant amount of water in a stable, hydrogen bonded complex\(^\text{27}\). In contrast, partitioning systems such as hexane/water and chloroform/water contain so little water in the organic phase that they are poor modes for the lipid bilayer/water found in the body.

Lipid soluble drugs usually cross cellular boundaries by dissolving in or interacting with the lipid membranes and diffusing across into the intracellular aqueous phase. Most drugs are weak organic electrolytes present in equilibrium between two forms, of which only the non-ionized form possesses lipid solubility.

Since our compound was found to have relatively low partition coefficient, it has as a result relatively low lipid solubility, and as a consequence it will poorly absorbed from the G.I.T after oral absorption.

In addition to that, it has relatively large molecular size, which is an additional factor for poor absorption. On the other hand our compound is a phosphate diester, and this type of conjugation is resistant to chemical cleavage by the G.I fluid.

So we expect that our compound will persist for a long period of time during which it will pass the G.I tract and reach the colon in a significant quantity to exert its local effect after complete hydrolysis and liberation of the active moieties.

The U.V. spectrum of the compound (0.1mg/ml distilled water) show \( \lambda_{max} \) at 320nm, \( \lambda_{max} \) for dexamethasone phosphate and metronidazole was found to be 243nm and 264nm respectively.
REFERENCES
Does Vitamin E Improve Hyperlipidemia in Patients with Cardiovascular Disease?

Received 17-10-2004 Accepted 5-6-2005

ABSTRACT

This study was carried out to evaluate the hypolipidemic activity of vitamin E in patients with cardiovascular disease. Fifty-nine patients aged 30-60 yrs with different cardiovascular diseases were participitated in this study. Fifteen healthy subjects were also included in this study as a base line control.

Vitamin E in a daily dose of 400 mg was given for all patients for three months. Blood samples were drawn after an overnight fast from patients and control before treatment and at one month interval for three months after treatment for the patients to measure lipid profile and malondialdehyde.

At base line lipid profile and malondialdehyde –MDA- levels were significantly higher in the patients than those of the control. With vitamin E the levels of serum cholesterol, low density lipoprotein –LDL- and MDA were decreased. These changes were progressive and significant at all the points throughout the study. Serum high density lipoprotein –HDL- level, however showed a gradual decline but significance was only achieved at the end of the treatment. Serum triglyceride levels were not significantly changed after treatment with vitamin E.

Our results suggest that vitamin E beside its antioxidant activity also has lipid lowering activity.

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INTRODUCTION

Vitamin E, a fat-soluble vitamin, protects vitamin A and essential fatty acids from oxidation in the body cells and prevents breakdown of body tissues \(^1\). The vitamin E appears to be the first line of defense against peroxidation of polyunsaturated fatty acids contained in cellular and subcellular membrane phospholipids \(^2\). It has been postulated that vitamin E has a role in the regulation of protein synthesis. Other actions of this vitamin also have been investigated, for example, effects on muscle creatine kinase and liver xanthine oxidase. Vitamin E deficiency leads to an increase in the turnover of creatine kinase. There is also an increase in liver xanthine oxidase activity in vitamin E deficient animals, and this increase is due to an increase in denovo synthesis \(^3\).

A review of the literature concerning the safety of oral intake of vitamin E indicated that the toxicity of vitamin E is low. Vitamin E supplementation has resulted in inconsistent effects in serum lipid and lipoproteins levels. Animal studies showed that vitamin E is not mutagenic, carcinogenic, or teratogenic. In human studies with double-blind protocols and in large population studies, oral vitamin E supplementation resulted in few side effects even at doses as high as 3200 mg/day \(^4,5\).

Vitamin E therapeutic indications include the clinical conditions characterized by low serum tocopherol levels and increased fragility of red blood cells to hydrogen peroxide or conditions that require additional amounts. The latter can be exemplified by individuals who consume excessive amounts of polyunsaturated fatty acids (more than 20 g/day) \(^6\).

It has been claimed that vitamin E could be of therapeutic benefit in ischemic heart disease, but evidence against this claim continues to accumulate. It also has been suggested that megadoses of tocopherol be used in the treatment of peripheral vascular disease with intermittent claudication \(^3\).

The scavenger receptor class B type I (SR–BI) mediates the selective uptake of cholesterol and cholesteryl ester (CE) from high density lipoprotein (HDL) into cells. Vitamin E exerts a tight control over the expression of SR–BI. In human hepatomen derived cells, the expression of the human SR–BI was reduced when the vitamin E content was increased by incubating the cells with vitamin E loaded HDL. The down regulation of human SR–BI (hSR–BI) was accompanied by a reduced level of protein kinase C (PKC) in the particulate fraction, and PKC inhibition decreased the expression of hSR–BI and the uptake of vitamin E and cholesterol from the view that the cellular level of HDL \(^7\).

Objective of the Study

To indicate the advantage of using vitamin E as hypolipidemic drug.

MATERIALS and METHODS

All patients gave informed consent before the study. Ninety five patients, ageing 35–60 years with different cardiovascular diseases (angina pectoris, old infarction and hypertension) were participated in this study. The patients were attending the out patient clinic at Ibn Al–Nafees Cardiovascular Teaching Hospital. They were obtained and approved by the ethical committee. Selection was based on laboratory findings of serum cholesterol and triglyceride levels. Any patients have serum cholesterol below 215 mg/100ml were excluded. Some of the patients were on thiazide diuretics and β-blockers.

Fifteen healthy subjects were included in the study as a control, with comparable age to the patients.

All patients received vitamin E in a dose of 400 mg once daily at night. Laboratory data (lipid profile, malondialdehyde-MDA), were done at base line before initiation of therapy and monthly after that (for three months) (table 2 and figures 1, 2, 3, 4, 5 and 6).

At baseline and on each visit (1, 2, 3 months), venous blood (10ml) was taken from the forearm of each patient by vein puncture. Blood samples were taken after blood pressure measurement and 12 hours fasting. Each sample was transferred to a plastic centrifuge tube. Sera were aspirated after centrifugation at 1000 rpm for 10 minutes and were analyzed for: Lipid profile (total cholesterol, triglyceride, and high density lipoprotein –HDL- and malondialdehyde (MDA)).

The sera were stored at –4°C for no longer than seven days, while serum MDA analysis was done within 24 hours after sample collection.

Lipid Peroxidation

Malondialdehyde formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index of peroxidation reaction. The thiobarbituric acid method of Buege and Aust (1978) was used to measure the malondialdehyde (MDA), which react with thiobarbituric acid (TBA) to give a pink color.
extinction coefficient of $1.5 \times 10^5$ $[^9]$. The results were expressed as $\mu$mol MDA / L serum.

**Serum Cholesterol**

Serum cholesterol was measured by cholesterol kit (ChoD–PAP) (Biomaghreb, Tunis) using an enzymatic method $[^10]$.

**Serum Triglycerides**

Serum triglycerides was measured by triglyceride kit (GPO–PAP) (Biomaghreb, Tunis) using the enzymatic method $[^11]$.

**Serum High Density Lipoprotein–Cholesterol**

Serum high density lipoprotein–cholesterol was measured by HDL kit PAP 150 (Biomaghreb, Tunis), using the enzymatic method $[^12, 13]$.

**Serum Low Density Lipoprotein–Cholesterol**

Serum low density lipoprotein–cholesterol (LDL–C) was calculated indirectly by using the Friedwald Equation $[^14]$,

$$LDL-C = \text{Total cholesterol} - \text{HDL-cholesterol} - \frac{\text{Triglycerides}}{5}$$

**Statistical Analysis**

Values are expressed as means ± SD. Student’s paired test was used for comparison of the parameters before and after treatment. A p value < 0.05 was considered statistically significant.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control level</th>
<th>Patients level</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. cholesterol (mg/100ml)</td>
<td>183.3 ± 21.3</td>
<td>233.8 ± 48.1*</td>
</tr>
<tr>
<td>Triglyceride (mg/100ml)</td>
<td>138.3 ± 50.3</td>
<td>188 ± 82.7*</td>
</tr>
<tr>
<td>HDL (mg/100ml)</td>
<td>48.3 ± 6.7</td>
<td>43.2 ± 10*</td>
</tr>
<tr>
<td>LDL (mg/100ml)</td>
<td>109 ± 20.3</td>
<td>161.6 ± 49.6*</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>2.3 ± 0.5</td>
<td>3.74 ± 2.1**</td>
</tr>
<tr>
<td>MDA $\mu$mol</td>
<td>0.083 ± 0.04</td>
<td>0.31 ± 0.08**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD
* P < 0.05.
** P < 0.005.

Table -2 shows that, there is significant decrease ($P < 0.005$) of serum total cholesterol levels after the 1st, 2nd, and 3rd month of treatment with vitamin E as compared to the base line level.

Table -2 : Effect of Vitamin E on Serum Levels of Lipid Profile (mg/100ml) and MDA ($\mu$mol/l).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Base line levels</th>
<th>After 1 month of treatment</th>
<th>After 2 months of treatment</th>
<th>After 3 months of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>233.8 ± 48.1</td>
<td>208 ± 47.4**</td>
<td>214.4 ± 57.5**</td>
<td>200.6 ± 52.8**</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>188 ± 82.7</td>
<td>196.1 ± 96.9</td>
<td>187.6 ± 70</td>
<td>185.5 ± 54.1</td>
</tr>
<tr>
<td>HDL</td>
<td>43.2 ± 10</td>
<td>39.9 ± 11.3</td>
<td>41.3 ± 9.6</td>
<td>34.5 ± 10.4**</td>
</tr>
<tr>
<td>LDL</td>
<td>161.6 ± 49.6</td>
<td>127.4 ± 48**</td>
<td>138.7 ± 57.7**</td>
<td>126.2 ± 49.9**</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>3.74 ± 2.1</td>
<td>3.2 ± 1.3**</td>
<td>3.3 ± 2.3*</td>
<td>3.6 ± 1.1**</td>
</tr>
<tr>
<td>MDA</td>
<td>0.31 ± 0.08</td>
<td>0.24 ± 0.05**</td>
<td>0.28 ± 0.07</td>
<td>0.24 ± 0.06**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SD
* P < 0.05.
** P < 0.005.
There are no significant differences in serum TG levels after 1, 2 and 3 months of treatment (table-2).

Serum HDL level shows a significant decrease (P < 0.005) after the 3rd month of treatment (table-2).

There is a highly significant decrease (P < 0.005) in serum LDL level after the 1st, 2nd and 3rd month of treatment compared to the base line level (table-2).

LDL/HDL ratio shows significant decrease (P < 0.005) after 1, 2 and 3 month of treatment compared to the base line (table-2).

Serum MDA level was decreased significantly (P < 0.005) after 1 and 3 month of the treatment course compared to the base line level (table-2).

**DISCUSSION**

Coronary heart disease (CHD) is one of the major causes of morbidity and mortality. A positive correlation has been demonstrated between raised serum lipids and the incidence of CHD and atherosclerosis in humans. Cholesterol is the lipid most frequently implicated in this relationship.[15]

In this study the patients who received vitamin E (400 mg/day) , (table-2) showed a significant decline in total cholesterol when compared with starting level at the end of the course therapy. The decline in cholesterol level by using vitamin E was significant. The results of this study agree with the results of other studies concerning the effect of vitamin E on plasma lipids. Khoja investigated the changes in the plasma lipid levels among rats fed an atherogenic promoting diet containing 0.5% cholesterol and rats fed the same diet with added vitamin C (ascorbic acid), vitamin E (α-tocopherol) and vitamins C + E from one to seven weeks. Rats supplemented with vitamin C, vitamin E or vitamin C + vitamin E for four to seven weeks showed significant decrease in the concentration of total cholesterol [16]. Thomas found that using vitamin E in mice fed a high-fat diet for 24 weeks was significantly lowered aortic cholesterol compared with control [17].

Heager[18] suggested that when vitamin E is maintained in excess of that provided by a normal diet in humans, it is beneficial in intermittent claudication and perhaps other manifestations of atherosclerosis. However, several workers showed that vitamin E may slow the progress of atherosclerosis by modifying the synthesis and degradation of lipids in the arterial wall and/or by increasing the proportion of blood cholesterol in the electrophoretic HDL–C fraction.[19]

Recently it had been reported that tocotrienols are form of vitamin E having an unsaturated side–chain, rather than the saturated side – chain of the more common tocopherols.[20]. Tocotrienols inhibit cholesterol synthesis by post–transcriptionally suppressing beta–hydroxy–methyl glutaryl–coenzyme A reductase activity [21]. Tocotrienols exert hypocholesterolemic action in humans and animals. Lovastatin is widely used for that purpose. Both agents work by suppressing the activity of beta–hydroxy–methyl glutaryl–coenzyme A reductase through different mechanisms, post–transcriptionally VS competitive inhibition[22]. It had been reported a significant reduction in serum total cholesterol and LDL–cholesterol using tocotrienol–rich fraction (TRF[23]) of rice bran alone and in combination with lovastatin. Substitution of TRF[24] with alpha–tocopherol produces significant changes when given with lovastatin. However, it can be suggested that vitamin E can act as a hypolipidemic agent like tocotrienol by post–transcriptionally suppressing β–hydroxy methyl glutaryl CoA reductase activity. In this study vitamin E failed to reduce TG significantly and although plasma HDL level of the patients of all groups was decreased (Table-2) but this reduction is less than that of LDL level in that LDL/HDL ratio which is significantly reduced. Many factors associated with low–HDL–cholesterol such as drugs (Thiazid diuretics, some beta–blockers) and diet (high polyunsaturated fat diet) [25] and most of these factors are found in our patients in that the patients were on normal diet and most of our patients have a chronic disease as hypertension, and angina, some of the drugs used in the treatment of these diseases are associated with low HDL level such as thiazid diuretic and beta blockers. All these factors may contribute together for the low level of HDL cholesterol. It was observed significantly lower concentration of LDL–C in the plasma of patients maintained with vitamin E(table 2). This is in agreement with the findings of Ram[26] who reported a significant decrease in serum LDL cholesterol level in experimentally induced hyperlipidemic rabbits using vitamin E alone and in combination of ethanolic extract (50 v/v) of plumbagozeylanica (root). It has been reported a significant reduction in LDL–C concentration in the plasma of patients with polycystic syndrome using a combination of metformin and vitamin E while that using metformin alone fail to reduce plasma LDL–C level[27]. The patients in our study showed a significant decline of the LDL–C /HDL–C ratio early after one month of the
treatment (table -2). Although HDL-C level in all patients were declined only after the third month, these results indicate that the treatments used in this study are beneficial in all patients.

Table-1 showed that all patients participated in this study have an oxidative stress since there is a significant difference between serum levels of MDA of these patients and controls. In this study the levels of MDA have shown to be decreased significantly after first month of treatment (table -2). Steinberg et al. reported that when LDL–C is in the modified or oxidized form, then it is favored for the formation of foam cells from monocytes /macrophages. Furthermore it has been shown that the addition of vitamin E to cell cultures blocks the oxidative modification of LDL [26].

The importance of oxidative modification of LDL-C and increased atherosclerosis, thrombosis and myocardial ischemic damage in leading to CHD may depend on the fatty acids composition of the diet. Poly–unsaturated fatty acids are vulnerable to free radical attack. Lipid peroxidation can become autocatalytic, but the chain reaction can be prevented by the action of vitamin E [27].

CONCLUSION
Vitamin E, beside its antioxidant ability, also have lipid lowering activity.

Recommendation:
The addition of vitamin E to statin may be of a great therapeutic advantage to the management of hyperlipidaemia.

REFERENCES


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Using Tissue Culture Technique  
for the Production of Cardiac Glycosides From Roots of Digitalis purpurea L Plantlets  
( Var. Excelsior Mixed )

Zeinab J. Aawad*, Ekbal H. Al-Khateeb* 
Received 10-7-2004 Accepted 5-7-2005 

ABSTRACT 
The present study was performed using the tissue culture technique, where by roots formation was stimulated shoot tips of Digitalis purpurea L. plant using auxins in this study. Shoot tips (1 cm length) were excised from sterilized seedlings of Digitalis purpurea L. Var. Excelsior mixed, then they were cultured on MS medium with the addition of one of the auxins IAA, IBA, NAA of different concentration (0.0, 0.05, 0.1, 0.5 and 1.0 mg/L). After 45 days of starting the culture results showed that the treatment 0.5 mg/L of IBA had a high significant effect on the dry weight of roots formation and containing Digitoxin and Gitoxin whose quantities as a rate of (2.96 gm, 30.01 and 11.05 μg/gm dry weight respectively. Also this treatment has given highest values with the other studied characteristics where the percent of the soluble sugars and starch and they are (2.48 %, 2.82%) respectively.

Abbreviation
(Indole Butyric Acid) IBA, (Indole Acetic Acid) IAA, (Murashige and skoog medium, 1962) MS, (Naphtalen acetic Acetic) NAA, (Milligram) Mg, (Microgram) μg, (Litter) L, (Gram) gm.

INTRODUCTION

Digitalis purpurea L is one of the herbal plants, belonging to the scrophularaceae family. This plant is considered to be one of horticultural plants and it is planted as an ornamental in the garden for its beautiful flowers and coordination as well as its various colours (1).

Digitalis purpurea has a great benefit in the medical and pharmacy fields because it contains cardiac glycosides which are found in all of its parts especially the leaves (2). These compounds are used to heal some of the important heart diseases like congestive heart failure (3). Because of their interest and demand in the medical field and due to the possibility of the
industrializing them in chemically or microbiologically, so the only method available to get these compounds is in the agriculture production (4). From this importance, many pharmacological, chemical and agriculture researches were carried out to study this plant in order to raise its contents for these compounds via using modern technologies like cell, tissue and plant organ culture (5), via the usage of this technology different explants can be cultured and stimulated to different growth in order to be a permanent source for these important medical compounds without being restricted to the environmental conditions as well as cleans of the pharmaco which could be obtained (6).

So the aim of the present study performed in the tissue culture field is to:

- stimulate the shoot tips of *Digitalis purpurea* plant to form roots by adding different kinds and concentrations of auxins to MS medium, and then we can benefit from the formed roots to be a source for demanded cardiac glycosides Digitoxin and Gitoxin.

**METHODS and MATERIALS**

The present study contained the following:

**First - Tissue Culture of the Plant *Digitalis purpurea***

The work went through the following steps:

1. **Initiation a tissue culture:**

   That is, initiating a specific culture empty of any contamination, so that it would be a source for shoot tips which are desired to be cultured and that was performed by preparing a sterile medium for culturing the seeds which contain merely distal water and agar (7), whereby difco – bacto – agar kind was used with 6 gm/L, which was added to distilled water.

   Then the heating process was performed till boiling with the use of magnetic stirrer hot plate machine, after that the medium was distributed in glass test tubes with a capacity of 25 x 180 ml and a quantity of 10 ml for every tube then they were covered with special coverlet and autoclaved in autoclave at a temperature of 121°C, pressure 1.04kg/cm², for 20 minutes, after disposing, then it was left to be cooled until the time of its culture. Next the seeds of the plant *Digitalis purpurea* variety excelsior mixed which was demanded for the culture is sterilized inside a laminar-air-flow cabinet with the 70% ethyl alcohol, for 30 sec, then rinsed with distilled sterilized water three times for 5 min for every time (8). Finishing this process the seeds were cultured and distributed upon the prepared medium surface. Then they were transmitted to the growth room under controlled - environmental conditions, 25 ± 2°C temperature, and photo period 16 hour/day with the severity amounting to 40 – 60 micro inshtain /m²/ sec. After 14 day, there were seedlings in which the demanded shoot tips in them were ready to be amputated and cultured.

2. **Formation the roots**

   This process was demanded:

   **A-Preparation the Nutrient Medium MS (table 1).**

   This was performed with the stock solution preparation, then the content was merged with the requested size and to which one of the auxins IAA, IBA, NAA were added in different concentrations (0.0, 0.05, 0.1 ,0.5 and 1.0 mg/L). The size is completed with distal water and the pH was adjusted to 5.5 with the used of 1 N NaOH and HCl. Next agar was added in a concentration of 8gm/L and solution it as in 1. These media were distributed into glass test tubes with capacity of 25 x 180 ml and quantity of 25 ml for every tube. The tubes were covered with special coverlet and sterilized in an autoclave at 121 °C, and a pressure of 1.04 kg/cm², for 25 min period of time. After disposing them, they were left to be cooled at room temperature until the nutrient media were solid ready to be used in culture.

   **B- Shoot tips** from seedlings were isolated and cultured with one tip in every tube and cultures were incubated under controlled environmental conditions as in I for 45 days for the shoot tips to roots.
Table (1): Composition of the MS nutrient medium *(9) *

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrate (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370</td>
</tr>
<tr>
<td>CaCl₂2H₂O</td>
<td>440</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>27.85</td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>37.25</td>
</tr>
<tr>
<td>MnSO₄.4H₂O</td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄.4H₂O</td>
<td>8.6</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.025</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Inositol</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxine – HCl</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine – HCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3000</td>
</tr>
</tbody>
</table>

Second - Preparation the roots for chemical analysis.

After finishing the assumed incubation period necessary for rooting in the culture media, the formed roots had the following characteristics:

1- Percent of the rooting.

2- Estimation of the dry weight. The roots mass was disposed into the culture tubes and washed with flowing water to get rid of the agar residues conflutinated to it, then wiped with clean cloth to get rid of the washing water, and then the root samples were differentiated and spread out on the filter paper, to be dried in the oven at 40°C until the weight affirmation *(10) *. Later samples were grinder to a hard powder and kept in paper sacks, in dry condition (closed disicator) and dark place.

3- The ratio percent for the soluble sugars and starch were estimated according to the Joslyn *(11)* method.

4- Extracting and purifying the cardiac glycosides, here used the Fujii et al., *(12)* method.

5- The qualitative and quantitative assay for the studied cardiac glycosides, were done by using high performance liquid chromatography (HPLC) following Braga et al., *(13)* method.

The data were arranged with the above studied characteristics (1, 2, 3, 5) following the complete randomize design in a factorial experiment *(14)*.

RESULTS and DISCUSSION

1- The Roots formation.

Table-2 showed that stimulated the shoot tips of Digitalis purpurea plant to form roots for the control treatment (0,0 mg/L) and for all the auxins concentrations which were used except 1 mg/L NAA concentration which had lead after 25 day of starting the culture to the death of the cultured shoot tips, where in this table showed should that the rooting ratio was 100% for the control treatment and for all the IAA concentrations and (0.01, 0.1, 0.5) mg/L concentrations for IBA, and (0.05, 0.1) mg/L for NAA, whereas 1 mg/L concentration for IBA and 0.5 mg/L concentration for NAA the ratio of rooting was decreased and significantly different from the other studied treatments.
Table (2): The effect of the interference between IAA, IBA, NAA and their concentrations on the rooting ratio for the shoot tips of Digitalis purpurea plantlets

<table>
<thead>
<tr>
<th>Concentration mg / L</th>
<th>Rooting %</th>
<th>Auxins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IAA</td>
</tr>
<tr>
<td>0.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.05</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>L.S.D 5%</td>
<td></td>
<td>20.03</td>
</tr>
</tbody>
</table>

***All the shoot tips are dead

Digitalis purpurea plantlets

stimulating root formation in tissue culture is usually controlled by many factors such as:- Presence of growth regulators auxins, whether it could be a natural auxin, that was, naturally found inside the plant tissue or added to the nutrient medium \(^\text{15}\). Its physiological effect then, would be increase in cell division and transformation to meristem cells. In this case adventitious root meristem will have cell division to form the adventitious roots \(^\text{16}\). In the results of our study mentioned in table 2, it was noticed that:

In the control treatment (non-addition of auxins to the nutrient medium), the rooting process happened this might be due to existence of the natural auxin inside the shoot tips used for culture, and it might be that, this natural auxin was present in a quantity that helped and stimulate root formation, or otherwise, to the nutrient medium MS used, which probably helped to made root growth and development like P element, which in turn will have a role in stimulating root formation, and N element which will help to increase internal auxin concentration because this element involved in auxin composition \(^\text{17}\). So, naturally internal auxin concentration increase will affect root formation. More over sucrose has a role in root formation because it will be a source of necessary energy for this process \(^\text{18}\). All what is mentioned above would be interlinked to affect stimulation of root formation. Similar to the result of root formation stimulation without any external additions for the nutrient medium appeared in previous studies upon Digitalis plant like the study performed by Awad et al. \(^\text{19}\), Brisa and Segura \(^\text{20}\).

As for auxins types and concentrations added to the MS nutrient medium, affecting to root formation, the reason might be due to what is mentioned above about control treatment as well as to the known role of these auxins in stimulating root formation.

In comparison to the results found by Schoner and Reinhard \(^\text{21}\), the difference considerably in this study lies in:

- The number of the roots formed as they were very numerous and slim, and greatly attached to one another as in figure 1, that’s why it was not possible to count them and measure their lengths, which may involve other characteristics, and when their data are taken, they will help us to maintain more clarification for the difference between effect of types and concentrations of auxins upon root formation, growth and development. Hence, some differences appeared in other characteristics which were cleared and would be mentioned in 2.

As for the high concentrations at which rooting ratios were decreased or cultured shoot tips were dead, the reason might be due to that these high auxin concentrations were encouraging to made an increase in ethylene construction in cultured explants tissue, consequently it will lead to the frustration in their growth and development \(^\text{22}\).

*** All the shoot tips are dead
2. The production of cardiac glycosides, soluble sugar and starch.

Table 3 shows that the auxins concentrations which were used to stimulate root formation, had a positive and significant effect on the production of cardiac glycosides compared with the control treatment. Which could be due to increase the auxin level inside the cultured explants. This might help to make more development in most of the cell components like vacuoles and mitochondria, which have a role in cardiac glycosides formation (23).

As for the other characteristics especially soluble sugars and starch (table 4), it was studied because of the references made by many other workers, showed a positive effect on production of the cardiac glycosides in tissue culture (24).

They have shown that these characteristics have a positive relation with the quantities formed from the cardiac glycosides.

The results of our study in table 4, showed that the auxins concentrations which stimulated root formation, had significant effect upon increase of the percent for the soluble sugar, starch and total dry weight compared with the control treatment. The general reason is due to the role of the known auxin in forming these compounds (7), so far the increase in auxin concentration inside cultured explant tissue, with the effect of added auxins, to the nutrient medium will lead consequently to make an increase in the results of the different metabolic processes inside cell, like sugars and starch formation and this will be reflected positively upon cardiac glycosides formation (23). All this made an increase in the dry weight of the roots formed.

Table 3 and 4 clarify that the IBA had a greater effect upon the studied characteristics in comparison to IAA and NAA, the reason might be due to the strong effect of IBA caused by its slow decomposition by the oxidative enzymes which had little poison, slow transference and then it stays near its place of addition. As for the IAA, it was distinguished for its weakness because of its high oxidation by light and some of the oxidative enzymes for auxin inside plant and more than IBA and NAA (25). As for 1mg/L of NAA which led to the death of cultured shoot tips, the reason might be due to the high poison which was known for NAA appearing within little concentration compared with IAA and IBA (22). The poisonous concentrations of auxins usually lead to release high quantities of ethylene which lead to quick and unregulated cell division and then to release the destructive enzymes for the cell components which consequently lead to their death (22).

Accordingly, in our experiment the treatment which consisted of IBA auxin in 0.5 mg/L concentration was the best regarding formation of dry weight of roots besides containing the studied cardiac glycosides (Digitoxin and Gitoxin) and soluble sugars and starch.
Table (3): The effect of the interference between the auxins IAA, IBA, NAA and their different concentrations on production of the cardiac glycosides in roots of *Digitalis purpurea* plantlets.

<table>
<thead>
<tr>
<th>CONCENTRATION (MG/L)</th>
<th>CARDIAC GLYCOSIDES (µG/G DRY WEIGHT)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Digitoxin</td>
<td>Gitoxin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Auxins</td>
<td>Auxins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAA</td>
<td>IBA</td>
<td>NAA</td>
</tr>
<tr>
<td>0.0</td>
<td>0.87</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>0.05</td>
<td>4.37</td>
<td>10.31</td>
<td>3.77</td>
</tr>
<tr>
<td>0.1</td>
<td>4.57</td>
<td>18.92</td>
<td>1.77</td>
</tr>
<tr>
<td>0.5</td>
<td>7.97</td>
<td>30.01</td>
<td>0.54</td>
</tr>
<tr>
<td>1.0</td>
<td>7.41</td>
<td>11.89</td>
<td>1.0</td>
</tr>
<tr>
<td>L.S.D. 5%</td>
<td>2.11</td>
<td>1.92</td>
<td></td>
</tr>
</tbody>
</table>

*** All the shoot tips are dead

Table (4): The effect of the interference between the auxins IAA, IBA, NAA and their different concentrations on the roots of *Digitalis purpurea* plantlets according to the contents (soluble sugars and starch) and total dry weight.

<table>
<thead>
<tr>
<th>CONCENTRATION (MG/L)</th>
<th>SOLUBLE SUGARS (%)</th>
<th>STARCH (%)</th>
<th>DRY WEIGHT (G)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Auxins</td>
<td>Auxins</td>
<td>Auxins</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>IAA 1.10</td>
<td>IBA 1.10</td>
<td>NAA 1.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAA 1.54</td>
<td>IBA 1.54</td>
<td>NAA 1.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IAA 0.19</td>
<td>IBA 0.19</td>
<td>NAA 0.19</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>1.28</td>
<td>1.41</td>
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<tr>
<td></td>
<td>1.61</td>
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<td>0.1</td>
<td>1.32</td>
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<tr>
<td></td>
<td>2.11</td>
<td>2.13</td>
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<td>1.27</td>
<td>1.38</td>
<td>1.31</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.36</td>
<td>2.48</td>
<td>0.14</td>
<td></td>
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<tr>
<td></td>
<td>2.07</td>
<td>2.82</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.81</td>
<td>2.96</td>
<td>0.59</td>
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<tr>
<td>1.0</td>
<td>1.91</td>
<td>2.15</td>
<td>1.72</td>
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<td></td>
<td>2.21</td>
<td>2.34</td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.72</td>
<td>1.93</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>L.S.D</td>
<td>0.07</td>
<td>0.12</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

*** All the shoot tips are dead
References:
Antimicrobial Activity And Phytochemical Investigation Of Tamarix Macrocarpa (Ehrenb.)Bge Wildly Grown In Iraq
Ali A. Al-Shamma*, Enas J. Kadhum*,Mustafa M.A. Al-Hiti*
Received   2-3-2002    Accepted 24-7-2005

ABSTRACT
Ethanolic extract of Tamarix macrocarpa was tested for its, in vitro, antimicrobial activity by an agar dilution - streak method against eleven microorganisms. The results showed that it is active against Staphylococcus aureus, Bacillus subtilis at the level of 1000 mcg / ml and against Candida albicans at the level of 500 mcg / ml. Phytochemical investigation indicated that this plant is rich with phenolic and Polyphenolic compounds, among which four compounds were identified by both Thin Layer Chromatography and High Pressure Liquid Chromatography.

The identified compounds were: Gallic acid, Isoferulic acid, Kampferol and Quercetin.

This is one of the most significant studies concerning the phytochemical investigation of this fractionation and characterization of the antimicrobial components.

INTRODUCTION
Tamarix macrocarpa (Ehrenb) Bge (Tamaricaceae) is an indigenous plant, widely distributed in Iraq. It is one of eleven species found in Iraq (1). Tamarix macrocarpa (TARFA, طرة ,arabic and Colloquial name) is a shrub or small tree with brown to blackish. Brown bark, found in saline swamps, flats along margin of sand dunes, salty west lands. It is distributed in the desert region of Iraq, especially on the irrigated alluvial plain near Hawija, Samawa, near Baghdad, Diwania, and Basra (1). It's also distributed in Syria, Palestine, Jordan, Sinai, Egypt, Kuwait, Iran, West Pakistan and Libya (1).

Literature survey on different Tamarix species revealed a number of publications which mostly report the presence of phenolic and polyphenolic compounds, together with their esters and glycosides derivatives (29).

In concern of Tamarix macrocarpa, only one general screening study (10) reported that an ethanolic extract of the plant have a wide range of antimicrobial activities.

Since no further phytochemical studies were done on this plant, this study deals with the phytochemical investigation as well as fractionation and characterization of the active components.
**EXPERIMENTAL**

**Plant Material:**

The plant material (aerial part) of Tamarix macrocarpa (Ehrenb) Bge was collected at the blooming stage during the months of February, March and April from local fields about 5 Km. West of Faluja (35 Km. West of Baghdad).

The plant material was identified by the Department of Pharmacognosy College of Pharmacy, University of Baghdad. And authenticated by the National Iraqi Herbarium, Botany Directorate at Abu-Ghraib.

**Extraction:**

Three Kilograms of the plant material, in moderately coarse powder, were extracted with 4 liters of petroleum ether (b.p. 60-80 °c) at room temperature for 48 hours. The petroleum ether extract was filtered and evaporated to dryness under reduced pressure at a temperature not exceeding 40°c (leaving a yellowish — white viscous residue (15gm.).

The plant material was then percolated with 6 liters of ethanol 95%, collecting the percolate every 24 hours, until complete exhaustion. The alcoholic extract was evaporated to dryness, to give a dark green residue (95 gm), designated as (F-1). F-1 then was macerated with enough quantities of ether. The ether soluble fraction was separated, reduced to 300 ml. by evaporation, and then partitioned with equal volume of 5% sodium hydroxide solution (three times), in a separatory funnel.

The basic solution — was separated and then acidified with hydrochloric acid (35%) to PH2, using hydroion paper as indicator. A precipitate was formed which was extracted with 300 ml. Of ether (two times) by a separatory funnel.

The ether layer then separated, dried with anhydrous sodium sulphate, filtered and evaporated to dryness to leave a blackish residue about (9.5 gm) designated as (F-2).

Maceration of the alcoholic extract (left after ether maceration) was continued with 300 ml. of a mixture of methanol and water (50% v/v); the insoluble fraction then was separated by filtration leaving a residue of about (20 gm) designated as (F-3). The filtrate was evaporated to dryness to give a dark residue about (50 gm) designated as (F-5).

Maceration of (F-5) with enough quantity of chloroform gave a white residue which upon separation by filtration turn dark brown in colour (35 gm) designated as (F-6). The soluble fraction was evaporated to dryness to give a dark brown residue (15 gm) designated as (F-7).

**Antimicrobial Testins:**

A general test for antimicrobial activity was carried for all crude fractions (F-1 - F-7) obtained by the fractionation method.

They were tested by an agar dilution — streak method (11) at a concentration of 1000 mcgm/ml against eleven microorganisms representing gram positive and Gram negative bacteria as well as yeast and fungi (table 1).

A precipitate was formed, which was extracted with ethyl acetate (200ml) by a separatory funnel. The ethyl acetate layer then was separated, dried with anhydrous sodium sulphate, filtered and evaporated to dryness to leave a blackish residue about (13 gm) designated as (F-3).

Maceration of the original alcoholic extract (which was insoluble in ethyl acetate) was continued using 300 ml. of a mixture of methanol and water (50% v/v); the insoluble fraction then was separated by filtration leaving a residue of about (20 gm) designated as (F-4). The filtrate was evaporated to dryness to give a dark residue about (50 gm) designated as (F-5).

Maceration of (F-5) with enough quantity of chloroform gave a white residue which upon separation by filtration turn dark brown in colour (35 gm) designated as (F-6). The soluble fraction was evaporated to dryness to give a dark brown residue (15 gm) designated as (F-7).
Table -1 Microorganisms used in this study

<table>
<thead>
<tr>
<th>MICROORGANISM</th>
<th>CULTURE TYPE</th>
<th>CLASSIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus subtilis</td>
<td>ATCC 10231</td>
<td>Gram Positive bacteria</td>
</tr>
<tr>
<td>Micrococcus latus</td>
<td>ATCC 9341</td>
<td>Gram Positive bacteria</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC 6536</td>
<td>Gram Positive bacteria</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>ATCC 25922</td>
<td>Gram Negative bacteria</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>Hospital isolate</td>
<td>Gram Negative bacteria</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 9027</td>
<td>Gram Negative bacteria</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>Hospital isolate</td>
<td>Gram Negative bacteria</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>ATCC 16404</td>
<td>Fungi</td>
</tr>
<tr>
<td>Microsporum gypseum</td>
<td>Hospital isolate</td>
<td>Fungi</td>
</tr>
<tr>
<td>Trichophyton mentagrophate</td>
<td>Hospital isolate</td>
<td>Fungi</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>ATCC 10231</td>
<td>Yeast</td>
</tr>
</tbody>
</table>

**Thin Layer Chromatography and High Pressure Liquid Chromatography**

The components of all of the designated fractions (F-1 → F-7) were examined by Thin Layer Chromatography using the following system:

Silica gel GF 254 as a stationary phase with two solvent systems: Chloroform: Methanol (90:10 v/v) and Benzene, Acetone (95:5 v/v). Ferric chloride T.S. solution was used as a spraying reagent (12). Some of the identified components by Thin Layer Chromatography were further approved by High Pressure Liquid Chromatography as shown in (Fig-1, 2, 3).

The HPLC apparatus consist of Lc-6A liquid chromatography (Koyota, Japan), equipped with Reodyne 7125, 20 ml injector (USA). A Shimadzu Spd-6A UV/visible, detector set at 280 nm, 0.8 a. u.f.s. Column C-18 ODS (250x4.6 mm D): flow rate 1.5ml/ min, mobile phase, methanol: water (7.5: 92.5 v/v):

Wavelength 254 nm: Pressure 150: temperature 25-30°

**RESULTS and DISCUSSION**

Literature survey revealed that all of the reported compounds isolated from different species of tamarix were phenolic and polyphenolic and their derivative compounds of different polarities.
Since most of the isolated antimicrobial and antifungal compounds of plant origin were phenolic and polyphenolic compounds our work was emphasized on the study of these compounds. For this reason our experimental procedure dealt with the fractionation of the polyphenolic compounds of different polarities. So the alcoholic extract fraction (F-1) was fractionated using solvents of ascending polarities, starting with ether (F-2), ethyl acetate (F-3), and then methanol (F-7). Each fraction was tested with FeCl₃ test solution for the presence of phenolic compounds, and then tested for antimicrobial activities at the level of 1000 mcg/ml by an agar dilution-streak method.

Although all of the fractions (F-1–F-7) showed a number of positive phenolic spots on TLC, they were all having no antimicrobial activities, with the exception of the alcoholic fraction (F-1), which showed activity at the level of 1000 mcg/ml against Staphylococcus aureus, Bacillus subtilis and Candida albicans, and (F-7) which contains the highly polar phenolic compounds. This fraction (F-7) showed activities against Staphylococcus aureus and Bacillus subtilis at the level of 1000 mcg/ml. And showed anti fungal activity against Candida albicans at the level of 500 mcg/ml.

Attempts to obtain pure antimicrobial component "were not successful due to its highly oxidizable properties and their instability in the solution, as the colour of the solution was changing during the procedure from brown, to blackish-brown then to black in colour.

Phytochemical investigation of the alcoholic extract (F-1) by TLC indicated the presence of not less than 15 different phenolic compounds. Of these four of them were identified. They are Gallic acid, Isoferulic acid, Kampferol and Quercetin.

Identity of these compounds with authentic samples were confirmed by Thin Layer Chromatography using two different solvent systems. Further more they were confirmed by High Pressure Liquid Chromatography as shown in (Fig-1, 2, 3).

HPLC of the original alcoholic extract of T. macrocarpa.
HPLC for the standard references

1. Gallic acid  
2. Rutoside  
3. Quercetol.

4. Isoflavone acid

5. Kamferol

6. Quercetin

HPLC for the standard references
REFERENCES


10. Soulian, Ahmed M.A. et al. " Phenolics from the bark of tamarix aphylla " phytochemistry 1991, 30 (11), 3763-6 (Eng.).

