In Vivo Effect of *Catharanthus roseus* Crude Extracts on Pathogenic Bacteria Isolated from Skin Infections

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**Abstract**

This study includes collection of 70 swabs samples of burns from patients were admitted in three hospitals (Baghdad, Al-Numaan and burns injuries Hospital). All swabs samples were cultured on blood and MacConkey agar media to isolate and identify pathogenic bacteria according to their morphological, biochemical and growth characters. Growth of bacteria on selective media showed the following results: *Pseudomonas aeruginosa* 44.28%, *Klebsiella pneumonia* 30%, *Staphylococcus aureus* 8.57%, *Escherichia coli* 4.28%, *Proteus vulgaris* 4.28%, *Enterobacter* spp. 5.71%, *Acinetobacter baumannii* 2.89%. Different concentrations were prepared from leaves ethanolic crude extract of *Catharanthus roseus*, then the anti-bacterial activity of this extract was evaluated in vivo. Burns were achieved experimentally in Albino mice and contaminated with pathogenic bacteria and evaluate the ability of extract in healing of these infected burns. Results showed that the extract accelerate burns healing within 7 days.

**Keywords:** Medicinal plants, antimicrobial crude extracts, wounds and burns healing, *Catharanthus roseus*

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تاثير مستخلصات نبات عين البزون *Catharanthus roseus* في البكتريا المرضية المعزولة من الالتهابات الجلدية داخل جسم الكائن الحي

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**الخلاصة:**

تتضمن هذه الدراسة 70 عينة من منسحات الحروق والتي تم جمعها من المرضى الراقدين في مستشفى بغداد التعليمي ومستشفى النعمان التعليمي ومستشفى اصابات الحروق. جميع العزلات تم زرعها على الأوساط الزراعية وهي وسط اكار الدم ووسط الماكونكي لعزلها وتشخيصها تبعا لصفاتها الزراعية والشبيهية والبايوكيميائية. أظهر النمو البكتيري على الأوساط الزراعية تفاوت النسب المئوية للنمو كل بكتريا وكالتالي: %44.28 *Pseudomonas aeruginosa*, %30 *Klebsiella pneumonia*, %8.57 *Staphylococcus aureus*, %5.71 *Escherichia coli*, %4.28 *Proteus vulgaris*, %2.89 *Enterobacter* spp., %2.89 *Acinetobacter baumannii*. تم تحضير تركيزات مختلفة من مستخلص الأوراق لزهرة عين البزون وتم تقييم فعالية هذا المستخلص ضد النمو البكتيري داخل الجسم الحي، وقد تم استخدام الفئران المختبرية وذلك بإجراء حروق

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Introduction:

Burns are one of the most common and devastating forms of trauma. Patients with serious thermal injuries require immediate specialized care in order to minimize morbidity and mortality. Data from the national center for injury prevention and control in the United States showed that approximately 2 million fires were reported each year which resulted in 1.2 million people with burns injuries[1]. Very young children and the elderly have an increased risk of being burned and worse clinical outcomes than patients in other age groups[2]. Medicinal plants play a major role as antimicrobial agents. The use of and search for drugs and dietary supplements derived from plants have accelerated in recent years which are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids. These compounds have been found in vitro to have an anti-microbial properties [3]. Since pathogenic bacteria become more resist to antibiotics, there for this study aim to isolate the bacterial species from contaminated burns and then evaluate the anti-bacterial activity of the Catharanthus roseus leaves extract against these bacteria in vivo by evaluate the effect of C. roseus extract by using albino mice which associated with skin that infected experimentaly with burns In vivo.

Materials and methods

Identification of bacteria

Bacteria were identified depending on the morphological features on blood and MacConkey agar media. Biochemical tests also used to identify bacterial isolates by Vitek -2- as in Figure-1.

![Figure 1- Instrument of Vitek -2-](image)

Vitek -2- compact system forms from two pants. Instrument and computer. The instrument forms from five parts:
2. Fill door in which the sample was transport from kan tube into the kit by transfer tube through 70 seconds.
3. Load Door in this part the transfer tube was cut from the kit and loading the latter into the incubator during 3-5 min.
4. User access door. In this part all changes which occur as a result of Bacterial growth were measured to give the end result.
5. Waste Door. In this part a collection of kit was done at the end of process. Vitek -2- was used to identify the bacterial isolates, sensitivity test and colonies counting.
Principle
Identification instrument contains 64 wells. Each well has drying media and color indicator to record all color changes which occur as a result of bacterial growth.

Preparation of isolate
Isolates of bacteria were cultured on Nutrient agar by streaking and incubated at 37°C for 24 hr. Kan tube was filled with 3ml of Bacterial suspension. Optical density of the suspension was measured by a Densi check and it was (0.5-0.63). After preparation of sample, it puts in the fill Door to transfer it from Kan tube to the kit. The last step is transfer the sample to the load Door by hand and incubated. Result was given during (4-6 hr.). Isolates of bacteria were also identified by using biochemical tests which were Gram stain, Oxidase test [4], Catalase test , Indol test, Methyl red [5], Voges-Proskauer test, Simmon’ citrate Utilization, Triple sugar iron agar[4], Motility test [6], Urease production [7].

Preparation of C. roseus crude extract
Collection of plant sample
The fresh leaves from C. roseus var. “alba” were collected from the garden of Karbalaa University during June, July and August 2012. The plant materials were washed thoroughly with tap water and then with sterilized distilled water. The plant materials were dried in shade at room temperature (25±2°C) to dry and used as raw materials for the extraction of antimicrobial compounds from the plant.

Preparation of plant extract
The extract were prepared by using dry leaves. 10 g was powdered by electrical blender. 100 ml of 70% ethanol was used for the extraction of 10 g in the Soxhlet apparatus. The plant material was loaded in the inner tube of the Soxhlet apparatus and then fitted into around bottomed flask containing 70% ethanol. The Solvent was boiled gently 40°C over a heating mantle using the adjustable rheostat. The extraction was continued for 8 hr. The solvent was removed at the reduced pressure with the help of Rotary Vacuum Evaporator to yield a viscous residue of leaves [8].

GC-MS analysis of ethanolic crude extract of C. roseus
GC-MS analysis was done according to Tokuşoğlu with some modifications [9]. Gas Chromatography – Mass Spectrometry GC-MS analysis used an Agilent 6890 GC system coupled with an Agilent 5973N MSD operating at 70 eV. ion source temperature 200 °c, in lit temperature 200 °c; split injection (1 μl injection volume, split ratio,50:1). Capillary column (HP-5MS 30 m x 0.25 mm ID x 0.25 μm film, Agilent J & W, USA) was used; oven: 100 °c/min ; 275 °c at 10 °C/min for 20 min; transfer line temp.: 220°C. Carrier gas helium; constant flow rate 1 ml/min; data acquisition by Agilent GC/MSD Chem-Station Version D.02.00

Preparation of leaves extract concentrations
Absolute crude of leaves extract was diluted with absolute alcohol to make different concentrations. These concentrations were (1, 2, 5, 7, 10, 12, 13 and 15) mg/ml and prepared by the law v1c1=v2c2.

Study the antibacterial effect of C. roseus crude extracts in vitro
Agar dilution method
(MIC) was also determined by using Agar dilution method (National Committee for clinical laboratory standards) (NCCLS, 1998) which involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium according to the law v1xc1 = v2xc2. These concentrations were (1, 2, 5, 7, 10, 12, 13 and 15 ) mg/ml. 0.5 ml of the standardize number of bacterial cells which in contact to McFarland were applied to the surface of agar plate in addition to the control of the agar plate without antimicrobial agent. All plates were incubated over night at 37°C.

Study the antibacterial effect of C. roseus crude extract on bacteria and burns healing in vivo
Six to eight weeks old Albino mice males (weighed 20-25 g) were used in this study. It housed in plastic cages under optimum conditions of food, water, light and temperature. All animals were assigned to the following experimental groups:
- A: control group with burns injuries, nine mice in this group.
- B: group of mice with burns injuries received prepared treatment, six mice in this group.

Transcutaneous 10 mm in length burns injuries were performed experimentally on the backs of the mice, 50μl of bacterial suspension (1.5x10^6 CFU/ml) of Pseudomonas aeruginosa which was isolated from patients with infected burns were swabbed to the burns of group B.
Treatment

Preparation of treatment

Two types of treatment were prepared as in the following:
1. Equal volumes of C. roseus crude leaves extract and petroleum gel which taken from Al-numeric hospital were mixed to obtain a concentration which was 90 mg /ml.
2. Equal volumes of C. roseus crude leaves extract and cream which taken from Al-numeric hospital to obtain a concentration which was 90 mg /ml.

Treatment of mice

Two days after experimentally burns were achieved and redness and abscess appeared. Mice were treated with prepared treatments as in the following:
1. Burns injuries of mice were treated with treatments which mentioned in above once daily for two weeks six mice were used in this treatment.
2. Burns injuries of mice in control group were treated with petroleum gel only (blank vaselin), and with cream only without plant extract, six mice were used in this treatment.
3. Burns injuries of mice in control group were treated also with Silverin (Silver sulphadiazine,1%), three mice ere used in this treatment.
4. Mice of control group were remained without treatment, three mice were used in this treatment.

All animals were killed. The surrounding tissue at the site of injury removed and histological sections were prepared [10]

Results and Discussion

Identification of pathogenic bacteria

The swabs were cultured on blood agar, MacConkey agar and eosin methylene blue. The bacterial isolates that appeared on MacConkey agar were identified according to their ability to ferment lactose. P. aeruginosa performed by it’s biochemical and cultural properties. It grows well at (37-42) °c; growth at 42 °c help to differentiate it from other Pseudomonas species in the fluorescent group. It is oxidase positive and does not ferment carbohydrates, but many strains oxidize glucose [11]. On blood agar P. aeruginosa colonies appeared blue-green while on MacConkey agar had a pale appearance because it’s non lactose fermenter. On EMB It showed good growth but no fermentation of sugars or acid production. For IMVIC test, it was (+ - - -). The rate of P. aeruginosa isolates which isolated from burns injuries was 44.28% [12].

Identification of bacterial isolates by vitek -2-

All bacterial isolates were identified by using vitek -2- according to the biochemical tests. All results which reported by vitek -2- were confirmed the identification of bacterial isolates as in Table-1.

GC-MS analysis of C. roseus ethanolic crude extract

GS-MS chromatogram of leaves extract study showed 17 peaks in C.roseus. The fragmentation patterns of the peaks were compared with that of the library of compounds. Leaves extract have 17 peaks with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) as in Table-2.

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>Test</th>
<th>Result</th>
<th>Test</th>
<th>Result</th>
<th>Test</th>
<th>Result</th>
<th>Test</th>
<th>Result</th>
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<th>Result</th>
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<td>APPA</td>
<td>_</td>
<td>ADO</td>
<td>_</td>
<td>PyrA</td>
<td>_</td>
<td>IARL</td>
<td>_</td>
<td>dCEL</td>
<td>_</td>
<td>BGAL</td>
<td>_</td>
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<tr>
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<td>BANG</td>
<td>_</td>
<td>AGLT</td>
<td>_</td>
<td>dGLU</td>
<td>_</td>
<td>GGT</td>
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<td>_</td>
<td>dMAL</td>
<td>_</td>
<td>dMAN</td>
<td>_</td>
<td>dMNE</td>
<td>_</td>
<td>BXYL</td>
<td>_</td>
<td>BAlap</td>
<td>_</td>
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<td>_</td>
<td>LiP</td>
<td>_</td>
<td>PLE</td>
<td>_</td>
<td>TyrA</td>
<td>_</td>
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<td>_</td>
<td>DSOR</td>
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<td>_</td>
<td>dTAG</td>
<td>_</td>
<td>DTRE</td>
<td>_</td>
<td>CIT</td>
<td>_</td>
<td>MNT</td>
<td>_</td>
<td>5KG</td>
<td>_</td>
</tr>
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<td>ilLATk</td>
<td>_</td>
<td>AGLU</td>
<td>_</td>
<td>SUCT</td>
<td>_</td>
<td>NAGA</td>
<td>_</td>
<td>AGAL</td>
<td>_</td>
<td>PHOS</td>
<td>_</td>
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<tr>
<td>GlyA</td>
<td>_</td>
<td>ODC</td>
<td>_</td>
<td>LDC</td>
<td>_</td>
<td>IHISa</td>
<td>_</td>
<td>CMT</td>
<td>_</td>
<td>BGUR</td>
<td>_</td>
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<tr>
<td>O129R</td>
<td>_</td>
<td>GGAA</td>
<td>_</td>
<td>IMLTa</td>
<td>_</td>
<td>ELLM</td>
<td>_</td>
<td>ILATa</td>
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</table>
Table 2 - Composition of *Catharanthus roseus* ethanolic leaves extract

<table>
<thead>
<tr>
<th>No</th>
<th>Compound</th>
<th>Retention time(min)</th>
<th>Amount (%)</th>
<th>Chemical formula</th>
<th>M.W.</th>
<th>Synonyms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cis-bicyclo[4,2,0]octa-37-diene</td>
<td>1.54</td>
<td>23.8%</td>
<td>C8H10</td>
<td>106</td>
<td>Bicyclo[4,2,0]octa-3,7-diene</td>
</tr>
<tr>
<td>2</td>
<td>Tartronic acid</td>
<td>1.70</td>
<td>76.0%</td>
<td>C3H4O5</td>
<td>120</td>
<td>Hydroxymalonic acid,Malonic acid-droxyhy,2-hydroxypropanedioic acid</td>
</tr>
<tr>
<td>3</td>
<td>Dimethyl sulfide</td>
<td>1.77</td>
<td>51.0%</td>
<td>C2H6S</td>
<td>62</td>
<td>Methyl sulfide,2,thiopropane,Dimethylthioether</td>
</tr>
<tr>
<td>4</td>
<td>Z-2-Dodecenol</td>
<td>4.5</td>
<td>76.0%</td>
<td>C12H24O</td>
<td>184</td>
<td>Z-2-Dodecen-1-o1, (2Z)-2-Dodecen-1-o1</td>
</tr>
<tr>
<td>5</td>
<td>DL-4,5-Octanediol</td>
<td>3.5</td>
<td>19.6%</td>
<td>C8H18O2</td>
<td>146</td>
<td>4,5-Octanediol</td>
</tr>
<tr>
<td>6</td>
<td>Hexadecane</td>
<td>5.56</td>
<td>6.78%</td>
<td>C16H32O4</td>
<td>226</td>
<td>n-cetane,n-Hexadecane,Cetane</td>
</tr>
<tr>
<td>7</td>
<td>Scyllo-inositol,1-C-methyl-</td>
<td>10.1</td>
<td>19.4%</td>
<td>C7H14O6</td>
<td>194</td>
<td>Mytilitol,Inositol,1-C-methyl-,Scyllo-,1-Methyl-1,2,3,4,5,6-cyclohexanehex -ol</td>
</tr>
<tr>
<td>8</td>
<td>Phthalic acid</td>
<td>11.0</td>
<td>11.6%</td>
<td>C21H32O4</td>
<td>348</td>
<td>No synonyms</td>
</tr>
<tr>
<td>9</td>
<td>Oxalic acid,allyl decyl ester</td>
<td>12.53</td>
<td>6.52%</td>
<td>C15H26O4</td>
<td>270</td>
<td>No synonyms</td>
</tr>
<tr>
<td>10</td>
<td>2-Propanol,1-(2-propenyloxy)-</td>
<td>12.6</td>
<td>14.1%</td>
<td>C6H12O2</td>
<td>116</td>
<td>2-P propanol, 1-(allyloxy)-1-(Allyloxy)-2-propanol</td>
</tr>
<tr>
<td>11</td>
<td>Phthalic acid,2-methoxyethyl propyl ester</td>
<td>12.8</td>
<td>15.9%</td>
<td>C14H18O5</td>
<td>266</td>
<td>No synonyms</td>
</tr>
<tr>
<td>12</td>
<td>Pentadecanal-</td>
<td>12.85</td>
<td>10.9%</td>
<td>C15H30O</td>
<td>184</td>
<td>No synonyms</td>
</tr>
<tr>
<td>13</td>
<td>1,2-Benzenedicarboxylic acid, butyl octyl ester</td>
<td>13.65</td>
<td>19%</td>
<td>C20H30O4</td>
<td>334</td>
<td>PX 914,Staflex BOP,Butyl octyl phthalate, Phthalic acid butyl octylester, PlasticizerOBP</td>
</tr>
<tr>
<td>14</td>
<td>Octasiloxane,1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexadecamethyloctasiloxane</td>
<td>14.14</td>
<td>41.8%</td>
<td>C16H50O7Si8</td>
<td>578</td>
<td>1,1,1,3,3,5,5,7,7,9,9,11,113,113,15,15-Hexadecamethyloctasiloxane</td>
</tr>
</tbody>
</table>
Effect of *C. roseus* ethanolic crude leaves extract in contaminated burns healing

The Histological sections showed different results as in the following. Control showing burn in mice’s skin without treatment with no significant changes and healing and loss details of epidermis layer was clear and loss of certain dermal appendages as it is showed in Figure-2.

![Figure 2](image1.png)

**Figure 2**- The section is showing a burn in the mice’s skin without treatment (control) H&E (250X)

The histological section of mice’s skin burn which was treated with petroleum gel only as a control still picture showing congestion of the blood vessels and chronic inflammatory cells (granulation tissue) as in Figure-3.

![Figure 3](image2.png)

**Figure 3**- The section is showing mice’s skin that was treated with petroleum gel only (control), H&E(250X)
Another section of burn in mice’s skin which was treated with silverin showing that the certain area of epidermis look like normal shape and structure and certain dermal structure in contrast with the control. These changes appeared in Figure-4.

![Figure 4](image1)

**Figure 4** - The section is showing a burn in mice’s skin that was treated with silverin, H&E(250X)

Burn in mice’s skin which was treated with *C. roseus* crude leaves extract mixing with petroleum gel was showing in Figure 5. The result revealed normal structure like appearance of the epidermis and also dermal appendages is near to the normal shape and structure in contrast with the control, this result agree with a research in which using *Carcica papaya* leaves extract in burn healing with (5%-10%) petroleum gel, the results documented the beneficial effect of plant with petroleum gel in acceleration of skin healing process in animals [13]. Also hair follicle is more clear and healing was most rapid by using the mixture of plant extract with petroleum gel (vaselin) than by using blank vaselin.

![Figure 5](image2)

**Figure 5** - The section is showing a burn in mice’s skin that was treated with *C. roseus* crude leaves extract mixing with petroleum gel, H&E (250x) for 7 days

The histological section of burn in mice’s skin which was treated with *C. roseus* crude leaves extract mixing with cream showing in Figure 6, ulceration in surface epithelial appeared cells and presence of crust, there is edema in the dermis and inflammatory reaction.
Figure 6- The section is showing a burn in mice’s skin that was treated with C. roseus crude leaves extract mixing with cream, H&E(250X) for 7 days.

From all the histological sections and results in above the better healing pattern with complete burns closure was observed in mice treated with prepared mixture of crude leaves extract with petroleum gel within 7 days Figure 5 while it took about (15-17) days in control mice which was treated with petroleum gel only Figure3, and about (18-20) days in control mice without treatment Figure 2. Results in this study agreed with Nayak and Pereira who used C. roseus extract in skin infection healing. The treatement of extract were found to epithelialize faster. C. roseus treated animals showed a significant reduction in the skin injury and epithelization period [14]. The preliminary phyto chemical analysis of the extract showed the presence of tannins and alkaloids [14]. Anyone of the observed phytochemical constituents present in C. roseus may be responsible for the healing activity. Recent studies have shown that phytochemical constituents like flavanoids and triterpenoids are known to promote the healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for skin contraction and increased rate of epithelialisation [14]. Results showed that C. roseus accelerate burns healing process. Mixture of petroleum gel with C. roseus extract showed good effect in burns healing.

References