ISSN: 0067-2904
GIF: 0.851

In Vivo Effect of *Catharanthus roseu*s Crude Extracts on Pathogenic Bacteria Isolated from Skin infections

Auhoud A.Almakhzumi[1*], Hind H.Aldulaimy[2], Abdullatif M. Jawaad[2]
[1]Ministry of health, Al-Numaan hospital, Baghdad, Iraq
[2]Department of Biology, College of Science, University of Baghdad, Iraq.

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 aeroginosa* 44.28% , *Klebsiella pneumonia* 30% , *Staphylococcu saureus* 8.57% , *Escherichia coli* 4.28% , *Proteus vulgaris* 4.28 % , *Enterobacter spp*. 5.71% , *Acinetobacter baumanni* 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 7days.

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

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

عهود عبد الستار المخزومي[١]*، هند حسين الدليمي[٢]، عبد اللطيف محمد جواد[٢]
[١]وزارة الصحة ، مستشفى النعمان التعليمي ، بغداد ، العراق.
[٢]قسم علوم الحياة ، كلية العلوم ، جامعة بغداد ، بغداد ، العراق.

الخلاصة:

تتضمن هذه الدراسة ٧٠ عينة من مسحات الحروق والتي تم جمعها من المرضى الراقدين في مستشفى بغداد التعليمي ومستشفى النعمان التعليمي ومستشفى اصابات الحروق. جميع العزلات تم زرعها على الاوساط الزرعية وهي وسط اكار الدم ووسط الماكونكي لعزلها وتشخيصها تبعا لصفاتها الزرعية والمظهرية والبايوكيميائية. اظهر النمو البكتيري على الاوساط الزرعية تفاوت النسب المئوية لنمو كل بكتريا وكالتالي: *Pseudomonas aeruginosa* بنسبة ٤٤,٢٨% ، *Klebsiella pneumonia* بنسبة ٣٠% ، *Staphylococcus aureus* بنسبة ٨,٥٧% ، *Escherichia coli* بنسبة ٤.٢٨% ، *Proteus vulgaris* بنسبة ٤,٢٨% ، *Enterobacter spp* بنسبة ٥,٧١% ، *Acinetobacter baumanni* ٢,٨٩%. تم تحضير تراكيز مختلفة من مستخلص الاوراق لزهرة عين البزون وتم تقييم فعالية هذا المستخلص ضد النمو البكتيري داخل الجسم الحي، وقد تم استخدام الفئران المختبرية وذلك باحداث حروق

*Email:iraqshams71@yahoo.com

في جسم الفئران ومن ثم تلويثها بالبكتريا المرضيةومعالجتها بالمستخلص المذكور اعلاه وتقييم فعالية هذا المستخلص وقد اتضح ان التئام الحروق باستخدام هذا المستخلص تم خلال ٧ ايام.

Introduction:

Burns are one of the most common and devastating form of trauma. Patients with serious thermal injuries require immediated 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 ages 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 flavonoides. 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-

Vitek -2- compact system forms from two pants. Instrument and computer.The instrument forms from five parts:

1. Keypad.
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 isoloate

Isolates of bacteria were cultured on Nutrient agar by streaking and incubated at 37^{o}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±2c^{o}) 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 40c^{o} 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^{o}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 $v_1c_1=v_2c_2$**.**

Study the antibacterial effect of *C. roseus* crude extracts *in vitr*o

Agar dilution method

(MIC) was also determined by using Agar dilution method (National Committee for clinical lab oratory standards) (NCCLS, 1998) which involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium according to the law $v_1 \times c_1 = v_1 \times c_2$. 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 contract 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^{o}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 (1.5×10^{8} 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-numaan 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-Numaan hospital to obtain a concentration which was 90 mg /ml.

Treatment of mice

Two days after experimentally burns were achieved and redness and abcess 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 mise ere used in this treatment.

4. Mice of control group were remained without treatmen, 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) ^{o}c; growth at 42 ^{o}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 1- Biochemical results by Vitek -2-

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
APPA	_	ADO	_	PyrA	_	IARL	_	dCEL	_	BGAL	_
H2S	_	BANG	_	AGLTp	_	dGLU	_	GGT	+	OFF	_
BGLU	_	dMAL	_	dMAN	_	dMNE	_	BXYL	_	BAlap	+
ProA	+	LiP	+	PLE	_	TyrA	_	URE	_	DSOR	_
SAC	_	dTAG	_	DTRE	_	CIT	+	MNT	+	5KG	_
iLATK	+	AGLU	_	SUCT	+	NAGA	_	AGAL	_	PHOS	_
GlyA	_	ODC	_	LDC	_	IHISa	_	CMT	_	BGUR	−
O129R	_	GGAA	_	IMLTa	_	ELLM	_	ILATa	_		

Table 2- Composition of *Catharanthus roseus* ethanolic leaves extract

No	Compound	Retention time(min)	Amount (%)	Chemical formula	M.W.	Synonyms
1	Cis-bicyclo{4,2,0} octa-37-diene	1.54	23.8%	C_8H_{10}	106	Bicyclo{4,2,0}octa-3,7-diene
2	Tartronic acid	1.70	76.0%	$C_3H_4O_5$	120	Hydroxymalonicacid,Mlon-icacid-droxyhy,2-hydroxypropanedioicacid
3	Dimethyl sulfide	1.77	51.0%	C_2H_6S	62	Methyl sulfide,2,thiapropane,Dimethylthioether
4	Z-2-Dodecenol	4.5	25.9.%	$C_{12}H_{24}O$	184	Z-2-Dodecen-1-o1, (2Z)-2-Dodecen-1-o1
5	DL-4,5-Octanediol	3.5	19.6%	$C_8H_{18}O_2$	146	4,5-Octanediol
6	Hexadecane	5.56	6.78%	$C_{16}H_{3O49}$	226	n-cetane,n-Hexadecane,Cetane
7	Scyllo-inositol,1-C-methyl-	10.1	19.4%	$C_7H_{14}O_6$	194	Mytilitol,Inositol,1-C-methyl-,Scyllo-,1-Methyl-1,2,3,4,5,6-cyclohexanehex--ol
8	Phthalic acid	11.0	11.6%	$C_{21}H_{32}O4$	348	No synonyms
9	Oxalic acid,allyl decyl ester	12.53	6.52%	$C_{15}H_{26}O_4$	270	No synonyms
10	2--Propanol,1 -(2-prppenyloxyl)-	12.6	14.1%	$C_6H_{12}O_2$	116	2-P ropanol, 1-(allyloxy)-1-(Allyloxy)-2-propanol
11	Pthalic acid,2-methoxyethyl propyl ester	12.8	15.9%	$C14H_{18}O5$	266	No synonyms
12	Pentadecanal-	12.85	10.9%	$C_{15}H_{30}O$	184	No synonyms
13	1,2-Benzenedicarboxylic acid,butyl octyl ester	13.65	19%	$C_{20}H_{30}O_4$	334	PX 914,Staflex BOP,Butyl octyl phthalate, Pthalic acid butyl octylester,PlasticizerOBP
14	Octasiloxane,1,1,3,3,5,5,7,7,9,9,1,11,13,13,15,15-Hexadecameth-yloctasiloxane	14.14	41.8%	$C_{16}H_{50}O7$ Si8	578	1,1,1,3,3,5,5,7,7,9,9,11,113,113,15,15-Hexadecamethyloctasilox-ane

15	1,1,1,3,5,5,5-heptamethyltri-siloxane	14.18	30.1%	$C_7H_{22}O_2Si_3$	222	Trisiloxane,1,1,1,3,5,5,5,-heptamethyl-
16	14-Heptadecanal	14.45	9.83%	$C_{17}H_{32}O$	252	No synonyms
17	Silicic acid,diethyl bis(trimethylsilyl) ester	14.65	**36.5%**	$C_{10}H_{28}O_4Si_3$	296	3,3-Diethoxy-1,1,1,5,5,5,-hexamethyltrisiloxane, Diethyl bis(trimethylsilyl) orthosilicate

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.

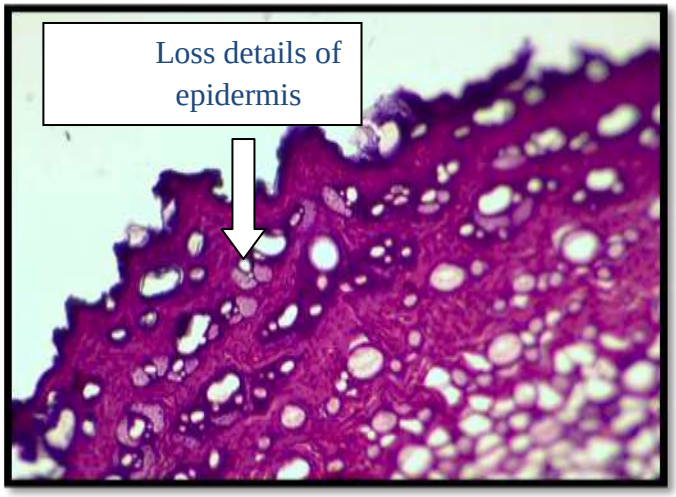

Figure 2- The section is showing a burn in the mice's skin without treatment (control) H&E (250X)

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.

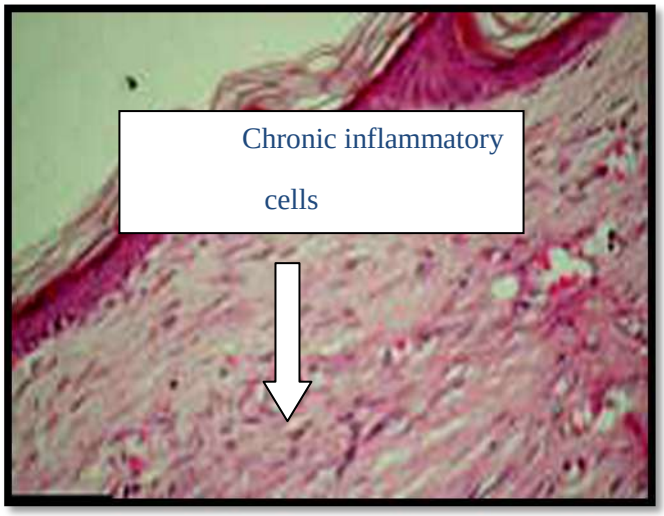

Figure 3- The section is showing mice's skin that was treated with petroleum gel only (control), H&E(250X)

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.

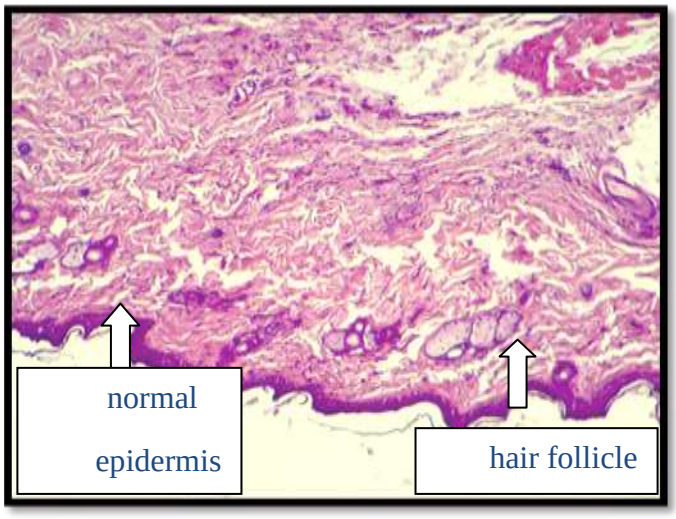

Figure 4- The section is showing a burn in mice's skin that was treated with silverin, H&E(250X)

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.

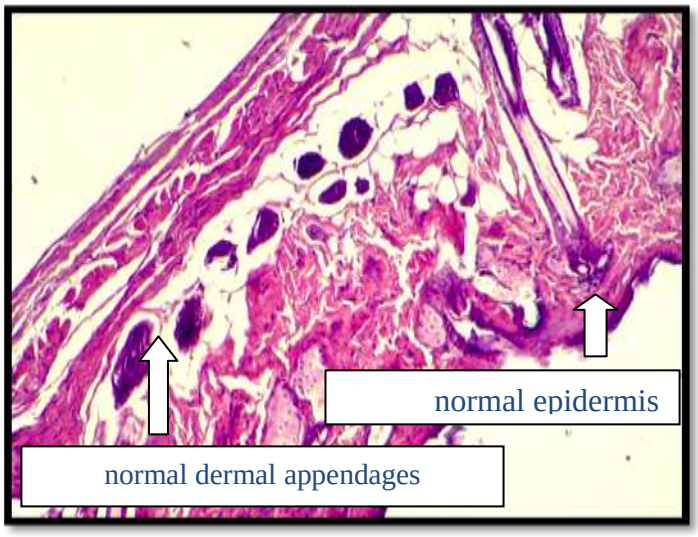

Figure 5- The section is showing a burn in mice's skin that was treated with *C. roseus* crude leaves extract mixing with petroleum gel, H&E (250x) for 7 days

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 crast, there is odema in the dermis and inflammatory reaction**.**

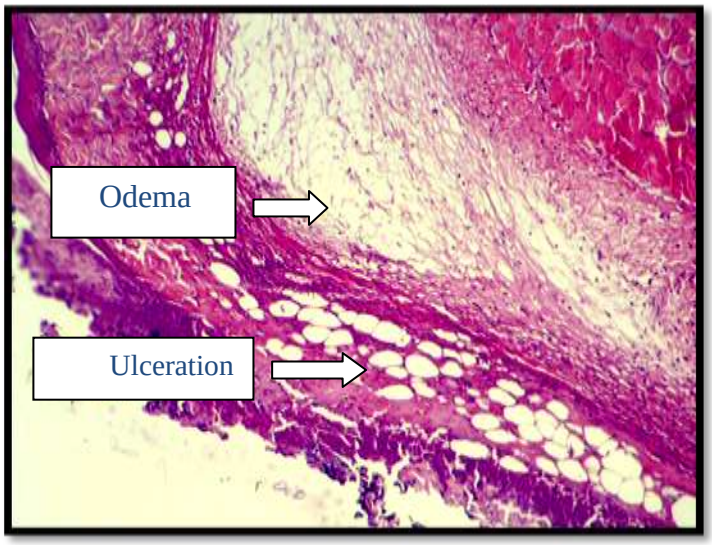

Figure 6- The section is showing a burn in mice's skin that was treated with *C. roseus* crude leaves extract mixing with cream, H&E(250X) for 7 days .

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 Figigure3, 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 extract-treated burn 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

1. Roth **,** J. J. and Hughes, W.B. **2004** . *The essential burn unit hand book*. Quality medical publishing 1st ed. Louis, London, England .
2. Pruitt, B. A., Goodwin, C.W. and Mason, J.R. **2002** . *Epidemiological Demographic and outcome characteristics of burn injury* , PP 16-30 . Total burn care. Saunders , London, England.
3. Cowan **,** M .M. **1999**. Plant products as antimicrobial agents. *J. clinical Microbiology Reviews* 12 (4), pp:564 – 582.
4. Harley, J.P. and Prescott, L.M. **2007**. *Labrotary Exercises in Microbiology*. 7th ed. Mc Graw – Hill Hinger Education New York.
5. Vasanthakumari**,** R. **2007**.*Textbook of Microbiology* . BI publication prt Ltd , New delhi.
6. Collee, J.G.: Fraser, A.G. Marmion , B. P. and Simmon , A. **1996**. *Mackie and McCartney medical microbiology*. 14th ed. The Chur. Living. Inc USA.
7. Macfaddian, J.F. **2000**. *Media for Isolation-Cultivation-Identification-Maintenance of Medical Bacteria*. Williams and Wilkins, Baltimore, USA MD.1, pp: 292-297.
8. Ramya, S. Govindaraji, V. Navaneetha Kannan, and K. Jayakumararaj R. **2008.** *In Vitro* evaluation of antibacterial activity using crude extracts of *Catharanthus roseus* L. (G.) Don. *Ethnobotanical Leaflets* 12, pp :1067-1072.
9. Tokusoglu,Ő., Őnal,MK. and yildirum, Z. **2003**. HPLC-UV and GC-MS characterization of the flvonol lagycons quercetin kaempferol and myricetin in tomato pasters and other tomato based products. *Acta Chromatographica,* pp:196-207.

10. Humason, C.L. **1972**. *Animal tissue techniques*. 3rd ed. W.H. Freem, New York.
11. Brooks ,G.F. ,Caroll , K.C. ,Butel , J.S., Mors , S.A., and Metzner , T.A. **2010**. Jawetz , *Melnick and Adelberg's Medical Microniology*.25th ed. The MCG raw- Hill companyUSA.
12. Nair, D.,Gupta,N.,Kabra, S., Ahuja, R.B. and Prakash, S.K. **1999**. *Salmonella* senftenberg: a new pathogen in the burn ward. *Burns*, 25,pp :723-727.
13. Mahmood , A.A. **,** Sidik , K. and Salmah, I. **2005**. Wound healing activityof *Carcica papaya* leaf extract in rats .*Journal of Molecular medicine and Advance sciences* ,1(4), pp : 398-401.
14. Nayak, B.S. and Lexley Pinto Pereira M. 2007 . *Catharanthus roseus* flower extract has wound-healing activity in Sprague Dawley rats, *J. Fitoterapia*, 78(7), pp:540-544.