Original Article

Evaluation of *Punica granatum* Peel Against Diabetic Wound Infection

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ABSTRACT

Background: Foot infections are a common and serious problem in diabetic patients.

Objectives: To investigate the antibacterial activity of some medicinal plants used by traditional healers for diabetic wounds and to examine in-vivo wound healing activity of active extracts.

Materials and Methods: An experimental study in which fifteen plant extracts subjected to preliminary antibacterial screening against six standard organisms (*Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa* and *Staphylococcus aureus*) using cup-plate agar diffusion method and the result was compared with activity of commonly used antibiotics. The minimum inhibitory concentration (MIC) was determined for active extracts using agar plate dilution method. The two extracts was screened against 180 clinical isolates obtained from diabetic wound infection. In this study the wound healing effect of methanolic extracts of *Punicagranatum* peels was tested on open skin wound model on Swiss Wistar Albino rats.

Results: Fourteen extracts (93.3%) exhibited inhibitory activity against one or more of the six organisms. The MICs of the methanolic and aqueous extracts of *Punica granatum* were very low against all organisms. 180 clinical isolates were obtained from diabetic wound infection, the results of identification showed that 30 were *Escherichia coli*, 15 *Klebsiella pneumoniae*, 45 *Proteus spp*, 15 *Pseudomonas aeruginosa*, 60 *Staphylococcus aureus* and 15 *Staphylococcus epidermidis*. The methanolic extract of *Punica granatum* peels had inhibitory effect against 99.4% of the isolates at concentration 100mg/ml. The aqueous extract has inhibitory effect against 91.7% of the isolates. Results were obtained by measuring the wound healing percentage. In the first group, healing was completed in 15 days. In the second group and third group, 13 days were required for the completion of healing.

Conclusion: The results of this study indicated that the methanolic extract of *Punica granatum* had high antibacterial activity and have wound healing activity.

Key words: Antimicrobial activity, wound healing, *Punica granatum*.

Diabetes mellitus has been a clinical problem for hundreds of years. Over 194 million people suffer from this disease worldwide [1]. Foot infections are a common and serious problem in diabetic patients. They usually occur as a consequence of skin ulceration, which is initially colonized with normal flora, and later infected with pathogens [2].Medicinal plants have been used as sources of medicine in virtually all cultures. During the last decade, the use of traditional medicine (TM) has expanded globally and is gaining popularity[3]. Up to 40% of modern drugs are derived from natural sources, using either the natural substance or a synthesized version[4].

The use of plants for healing purposes is getting increasingly popular as they are believed to be beneficial and free from side effect. However, the rationale for the utilization of medicinal plants has rested

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largely on long-term clinical experience with little or no scientific data on their efficacy and safety[5].

Punica granatum (Punicaceae) is used for treatment of many diseases by traditional healers. A decoction of seeds is used to treat syphilis. Juice is used to treat jaundice and diarrhea. Juice of flower is used to treat nosebleeds. The fruit peel is used for diabetic wound infection.

MATERIAL AND METHODS

Study area:

This research was conducted in Medicinal and Aromatic plants Research Institute (MAPRI), National Center for research.

Study population:

Clinical isolates were obtained from diabetic wound infection, collected randomly from Jaber Abu Aliz Diabetic Center.

Plant materials:

The plants used in this study were collected from different parts of Sudan. *Punica granatum* was collected from local market. They were authenticated by the Taxonomist Dr. Haider Abdelgadir and Dr.Wail Elsadig Abdalla, Medicinal and Aromatic plants Research Institute (MAPRI).

Preparation of Crude Extracts:

Fifty grams of dried powder sample was taken and extracted by Soxhlet apparatus using chloroform and methanol. The solvents were removed under reduced pressure in a rotary evaporator until they become completely dry. The residues were stored at 4°C for further use. Each residue was weighed and the yield percentage was determined and kept in refrigerator until used.

For aqueous extract 100 g of each plant sample was infused in 500 ml hot water for 4 hours then filtered with Whattman filter paper. Extracts were kept in Deep freezer at - 4°C for 48 hours, then introduced in freeze dryer till completely dried. The residue was weighed and the yield percentage was determined and kept in refrigerator until used.

Preparation of the test organisms:

One hundred and eighty clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*and *Staphylococcus epidermidis* were collected randomly from Jaber Abu Aliz Diabetic Center. The clinical isolates were obtained from diabetic wound infections. The purified isolates were identified by microscopical examination, cultural characters and biochemical tests and then stored in a refrigerator until they were used.

Preparation of bacterial suspensions:

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline, to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used.

Bacterial microorganisms:

Bacillus subtilis (Gram + ve Bacteria) ……NCTC 8236

Escherichia coli (Gram -ve Bacteria) ……ATCC 25922

Kelebsiella pnemoneae (Gram -ve Bacteria) ……ATCC 53657

Proteus vulgaris(Gram -ve Bacteria) .....ATCC 6380

Pseudomonas aeruginosa (Gram -ve Bacteria)..... ATCC 27853

Staphylococcus aureus (Gram +ve Bacteria).....ATCC 25923

National Collection of Type Culture (NCTC), Colindale, England.

American Type Culture Collection (ATCC) Rockville, Maryland, USA.

Experimental animals:

Swiss Wistar Albino rats of either sex, weighing 80-100g were used. Animals were supplied by the National Experimental Animal House (NEAH), Medicinal and Aromatic Research Institute (MAPRI), National Center for Research (NCR), Ministry of Science and Technology (MOST), Sudan. Rats were housed individually in a ventilated Animal house before and after surgery.

Testing for antibacterial Activity:

The cup-plate agar diffusion method [6] was adopted with some minor modifications to

assess the antibacterial activity of the prepared extracts.

One ml of the standardized bacterial stock suspension 10^8 –10^9 C.F.U/ ml were thoroughly mixed with 100ml of molten sterile nutrient agar which was maintained at 45 ºC. 20ml aliquots of the inoculated nutrient agar were distributed into sterile Petri-dishes.

The agars were left to set and in each of these plates 4 cups (10 mm in diameter) were cut using a sterile cork borer (No. 4) and agar discs were removed.

Alternate cups were filled with 0.1 ml sample of each extracts using automatic microlitre pipette, and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37 ºC for 18 hours.

Two replicates were carried out for each extract against each of the test organisms. After incubation the diameters of the resultant growth inhibition zones were measured, averaged and the mean values were tabulated.

Using the standard cup plate agar diffusion technique, the clinical strains were examined for susceptibility to the extracts which showed activity against standard bacterial organisms.

Determination of minimum inhibitory concentration (MIC) by agar plate dilution method:

The principle of the agar plate dilution is the inhibition of growth on the surface of the agar by the plant extracts incorporated into the medium. Plates were prepared in the series of increasing concentrations of the plant extract. The bottom of each plate was marked off into 6 segments. The organisms tested were grown in broth over night to contain 10^8 C.F.U/ml. Loop-full of diluted culture is spotted with a standard loop that delivers 0.001 ml on the surface of segment. The end point (MIC) is the least concentration of antimicrobial agent that completely inhibits the growth. Results are reported as the MIC in mg/ml.

Evaluation of extracts for wound healing properties:

The wound evaluation model [7] was adopted with some minor modification to assess the *in vivo* antimicrobial activity of a selected plant extract.

Full thickness wounds were made in the skin of the tested animals. Hair of the lower back and right flank of animals was fully shaved. Rats were lightly anaesthetized by inhalation using halothane. The animals were held in standard crouching position, and the mobile skin of flank was gently stretched and held by fingers. A metal circular object measuring 1 cm in diameter was placed on stretched skin and an outline of the object was traced on the skin using a fine tipped pen. The wound was made by excising the skin within the border of the object to level of loose subcutaneous tissue, using sterile forceps and scalpel blade. The artificial wounds were circular with a diameter of 1 cm.

The first day of the experiment was regarded as Zero. Animals were divided into three groups, each containing five animals:

Group 1 (wounded only):

Group 2 (wound +Tetracycline ointment 3%):

Group 3 (wound + MeOH extract of *Punicagranatum*5% in PEG):

Evaluation method of wound healing percentage:

In order to determine the rate of wound healing, every 24 hours, each animal was held in the standard crouching position and two diameters of the wound circle (horizontal and vertical) were measured using a transparent ruler. Measurement errors were minimized by repeating each measurement three times at the same moment and using an average of the calculations. The area of the wound in day zero was considered as 100% and the wound areas on subsequent days were compared with the wound on the day zero. Healing percentage in a certain day was the difference between the initial wound (in day zero) and healing wound on that certain day[8].

RESULTS

Identification of clinical isolates:

On the basis of the results of this identification test, it was found that the 180 clinical isolates were distributed as follows: 30 *Escherichia coli*, 15 *Klebsiellapneumoniae*, 45 *Proteus spp*, 15

Pseudomonas aeruginosa, 60 *Staphylococcus aureus*a and 15 *staphylococcus epidermidis.*

A Preliminary screening for antibacterial activity of some medicinal plants:

In the preliminary screening a total of 15 plant extracts belonging to 5 medicinal plants distributed among 5 families used by herbalists for diabetic wound healing, were screened for their antibacterial activity using cup-plate agar diffusion method. The results of this preliminary screening are summarized in table (1).

Determination of the Minimum Inhibitory Concentrations (MICs):

The minimum inhibitory concentration (MICs) of the methanolic and aqueous extracts of *Punica granatum* peel was determined against the standard organisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) as seen in table (2).

Antibacterial activity of reference drugs against standard organisms:

The antibacterial activity of selected reference drugs against standard organisms was determined using four different concentrations. The results are shown in table (3).

Antibacterial activity of methanolic and aqueous extracts against clinical isolates:

Table (4) shows the most active extracts methanolic and water extracts of *Punica granatum* was screened against the 180 clinical isolates at a concentration of 100 mg/ml using cup-plate agar method.

***In vivo* activity results of *Punica granatum* fruit peels:**

Results were obtained by measuring the wound healing percentage of the three groups of rats in this trial. In the first group, healing was completed in 15 days. In the second group and third group, 13 days were required for the completion of healing as illustrated in Table (5) and figure (1).

DISCUSSION

The current study was carried out to investigate the antibacterial activity of some medicinal plants used by traditional healers for diabetic wounds and to examine in-vivo wound healing activity of active extracts.

The commonest organisms isolated from diabetic wounds were *Staphylococcus aureus* (33.3%), *Proteus spp.*(25%) and *Escherischia coli* (16.7%).

Foot ulcers and their consequent infections are a common and serious cause of morbidity in patients with diabetes. *Staphylococcus aureus* is the most common isolate in these infections; the increasing incidence of methicillin-resistant *S. aureus* over the past two decades has further complicated antibiotic treatment. While chronic infections are often polymicrobial, many acute infections in patients not previously treated with antibiotics are caused by a single pathogen, usually a gram-positive coccus[9].

Among 21 diabetic patients with foot ulcer *Proteus mirabilis* was the most frequent microorganism recovered, and was regularly associated with *Staphylococcus aureus*. All the microorganisms isolated showed high sensitivity to second-generation quinolone antibiotics and were regularly sensitive to aminoglycoside antibiotics[10].

In the preliminary screening for antibacterial activity five medicinal plants (*Grewia senegalensis*, *Kigelia Africana, Punica granatum, Vitellaria paradoxa* and *Waltheria indica)* were examined against six standard organisms (*Bacillus subtilis, Escherichia coli, klebsiella pneumoniae, Proteus vulgaris, Pseudomanas aeruginosa* and *Staphylococcus aureus*). The total number of extracts were 15 extracts. Out of those extract, 14 (93%) exhibited inhibitory activity against one or more of the six organisms and one extract (6.7%) had no activity against the six standard organisms.

A total of seven antibiotics, Ciprofloxacin, Gentamycin, Vancomycin, Kanamycin, Amikacin, Co- Amoxiclavs and Erythromycin were tested at concentrations of 40, 20, 10 and 5 µg/ml against the six standard organisms. The result obtained indicated that ciprofloxacin showed the highest activity against the Gram negative and Gram positive organisms at the four different concentrations.

Table 1: The Antimicrobial activity of plant extracts against the Standard Organisms

Family/*Botanical*/ Vernacular/ Name	Part Used	Solvent	Yield %	Conc. Used/ml	Standard tested organisms* /M.D.I.Z (mm)**					
					B. s	*E. coli*	*K. pn*	*Pr.v*	*Ps.a*	*S. a*
Combretaceae *Grewia senegalensis* Gubeish	Leaves	MeOH	19	100mg	24	14	14	16	15	19
		$CHCl_3$	5.4	100mg	20	17	18	18	18	20
		H_2O	6.6	100mg	17	15	12	12	12	15
Bignoniaceae *Kigelia africana* Umm Mashttour	Fruits	MeOH	21.6	100mg	-	11	14	12	12	11
		$CHCl_3$	1	100mg	15	-	14	11	14	15
		H_2O	6.4	100mg	13	-	14	-	-	-
Punicaceae *Punica granatum* Roman	Fruits peel	MeOH	25.5	100mg	32	32	26	28	28	30
		$CHCl_3$	2.8	100mg	14	12	18	-	17	15
		H_2O	5.3	100mg	29	30	24	25	22	28
Sapotacea *Vitellaria paradoxa* Lolo	Fruits	MeOH	3	100mg	22	20	22	18	15	21
		$CHCl_3$	15.6	100mg	-	-	-	-	-	-
		H_2O	3.4	100mg	15	15	13	15	11	17
Sterculiaceae *Welthariaindica* IrgElnar	Roots	MeOH	10.7	100mg	30	18	22	19	21	22
		$CHCl_3$	1.4	100mg	18	14	14	15	17	17
		H_2O	2.5	100mg	20	15	14	14	17	18

*Standard organisms tested: *B.s. = Bacillus subtilis, S.a. =Staphylococcus aureus, E.c. = Escherichia coli, Pr.v. = Proteus vulgaris, Ps.a. = Pseudomonas aeruginosa,*
**M.D.I.Z=: Mean diameter of growth inhibition zone in (mm). MeOH=Methanol. $CHCl_3$=chloroform. H_2O= water

Table 2: The Minimum Inhibitory Concentration (MIC) of *Punica granatum* peel extracts against standard organism

Standard microorganisms	MIC of *Punica granatum* extracts	
	Methanol	Water
Bacillus subtilis	3.125mg/ml	3.125mg/ml
Escherichia coli	12.5mg/ml	25mg/ml
Klebsiella pneumoniae	25mg/ml	25mg/ml
Proteus vulgaris	6.25mg/ml	12.5mg/ml
Pseudomonas aeruginosa	12.5mg/ml	12.5mg/ml
Staphylococcus aureus	6.25mg/ml	6.25mg/ml

The methanolic and aqueous extracts of *Punicagranatum* showed activity against *Bacillus subtilis* and *Escherichia coli* higher than 40 µg/ml of all antibiotics tested. The methanolic extract inhibited *Klebsiella pneumoniae* higher than 10 µg/ml of Ciprofloxacin. It inhibited *Proteus vulgaris* and *Pseudomanas aeruginosa* similar to 5 µg/ml of Ciprofloxacin, and inhibited *Staphylococcus aureus* similar to 20 µg/ml of Co- Amoxiclavs.

The aqueous extract inhibited *Proteus vulgaris* similar to 40 µg/ml of Co-Amoxiclavs. The inhibition of the *Staphylococcus aureus* by the methanolic extracts of *Waltheria indica* was similar to 40 µg/ml Erythromycin and that of *Proteus vulgaris* was similar to 5 µg/ml of Co-Amoxiclav. For *Escherichia coli* and *Klebsiella pneumoniae* the zone of inhibition was equivalent to that obtained with 5 µg/ml of Ciprofloxacin. The extracts showed high activity against *Pseudomanas aeruginosa* better than Kanamycin, Amikacin and Gentamycin. The aqueous extract of *Waltheria indica* inhibits *Staphylococcus aureus* similar to 20µg/ml of Gentamycin, 5 µg/ml of Vancomycin and Erythromycin. It inhibits *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus vulgaris* similar to 40 µg/ml of Amikacin while it inhibits *Pseudomanas aeruginosa* similar to 40 µg/ml of Gentamycin. Khan and Hanee studied antibacterial properties of *Punica granatum* pericarp (peels) extracts (hot aqueous, methanolic and ethanolic) against *E.coli, P.aeruginosa and S.aureus* using agar well diffusion method. Hot aqueous, methanolic and ethanolic extracts of *Punica granatum* pericap show an average inhibitory zone diameter of 23.3, 22.3 and 24.5 mm respectively which indicates that ethanolic extract shows best result having ZOI greater than that of the standard antibiotic Tetracycline (20.1mm) [11].

The methanolic and water extracts of *Punica granatum* was the most active extracts among all the extracts tested. These extracts were studied against clinical isolates. The two extracts were screened against 180 clinical isolates recovered from diabetic wound infection.

The methanolic extract of *Punica granatum* peel had inhibitory effect against 99.4% of the isolates at concentration of 100 mg/ml. The aqueous extract has inhibitory effect against 91.7% of the isolates at the same concentration.

Petroleum ether, chloroform, methanol and water extracts of *Punica granatum* were studied by Prashanth *et al* for their antibacterial activity (in vitro). The methanolic extract was found to be most effective against all tested microorganisms and this is in agreement with this study[12].

The antibacterial activity of acetone, methanol and water extracts of pomegranate (*Punica granatum*) peels screened against Gram-positive and Gram-negative bacteria, acetone extract showed the highest antibacterial activity, followed by methanol and water extract[13].

In this study the wound healing effect of *Punica granatum* peel methanolic extracts was tested on open skin wound model on rats. Ointments of 5% (w/w) extracts in polyethylene glycol were prepared. Tetracycline ointment 3% was used as standard control. The healing period in the groups treated with methanolic extracts of *Punica granatum* peels ointment was 13 days as same as result obtained with Tetracycline 2% while the healing in the control group was completed in 15 days. This indicated that the two extracts were potent wound healing agents. The methanolic extract of dried pomegranate (*Punica granatum*) peels was formulated as a 10% (w/w) water-soluble gel and was studied by Murthy *et al* for its wound healing property against an excision wound on the skin of Wistar rats. The activity was compared with that of a commercial topical antibacterial applicant. The group of rats that received 5.0% gel showed complete healing after 10 days, whereas in rats treated with 2.5% gel, healing was observed on day 12, in contrast to the positive control animals receiving the blank gel, which took 16-18 days for complete healing [14].

Table 3: Antibacterial Activity of reference drugs against standard organisms

Drug	Concentration use µg /ml	*Standard organisms used /**MDIZ (mm)					
		B.s	*S.a*	*E.c*	*K.p*	*Pr.v*	*Ps.a**
Amikacin	40	19	19	16	18	15	15
	20	18	18	14	18	14	14
	10	17	17	12	18	11	11
	5	15	15	-	-	-	-
Co-Amoxiclav.***	40	12	35	15	-	25	-
	20	12	30	-	-	23	-
	10	-	25	-	-	20	-
	5	-	23	-	-	19	-
Erythromycin	40	23	23	-	-	-	-
	20	20	20	-	-	-	-
	10	20	20	-	-	-	-
	5	19	18	-	-	-	-
Ciprofloxacin	40	24	27	25	30	35	35
	20	21	27	23	28	35	32
	10	21	27	20	25	30	32
	5	20	25	20	24	30	29
Gentamycin	40	17	19	14	16	13	18
	20	17	18	13	13	12	15
	10	15	16	11	-	-	-
	5	15	16	11	-	-	-
Kanamycin	40	24	26	15	-	15	-
	20	22	24	14	-	15	-
	10	20	18	11	-	12	-
	5	16	18	11	-	12	-
Vancomycin	40	20	20	-	-	-	
	20	18	20	-	-	-	
	10	19	20	-	-	-	
	5	17	18	-	-	-	

*Standard organisms tested: *B.s. = Bacillus subtilis*, *S.a. = Staphylococcus aureus, E.c. = Escherichia coli*, *Pr.v. = Proteus vulgaris*, *Ps.a. = Pseudomonas aeruginosa*. **MDIZ : Mean diameter of growth inhibition zone in (mm).*** Co-Amoxiclav = Amoxicillin+ clavulanic acid. Interpretation of results: MIZD (mm) : >18 mm : Sensitive, 14 – 18 mm: Intermediate, < 14 mm: Resistant, –: No inhibition zone

The methanolic extract of *Punica granatum*, in the form of an ointment with two different concentrations (10% and 15% w/w ointment of extract in simple ointment base) was evaluated by Neema *et al* for wound healing potential in an excision wound model in rats. The results were comparable to standard drug Nitrofurazone ointment. It was observed that the wound contracting abilities of 10% and 15% extract ointments (97.8%, 98.4%) were greater than that of the control. The wound closure time was less and the percentage of wound contraction was much more with the 15% w/w extract ointment treated group. On 18th day 100% contraction was observed which was almost similar to that of the nitrofurazone ointment group[15].

Table 4: The activity of *Punicagranatum* fruit peels against clinical isolates

Organism	MeOH			H2O		
	Active	Moderate	Resistant	Active	Moderate	Resistant
E.coli	96.7%	3.3%	0%	83.3%	16.7%	0%
K.pneumoniae	66.7%	33.3%	0%	53.3%	26.7%	20%
Protus spp.	84.4%	15.6%	0%	46.7%	37.8%	15.5%
Ps.aeruginosa	80%	13.3%	6.7%	73.3%	20%	6.7%
S.aurues	86.7%	13.3%	0%	71.7%	25%	3.3%
S. epidermidis	80%	20%	0%	73.3%	20%	6.7%

Total No. of clinical isolates =180, *Escherichia coli* clinical isolates = 30, *Klebsiella pneumoniae* clinical isolates = 15, *Proteus spp*. clinical isolates = 45, *Pseudomonas aeruginosa* clinical isolates = 15 *Staphylococcus aureus* clinical isolates= 60, *Staphylococcus epidermidis* clinical isolates = 15
Interpretation of sensitivity results**:** (MIZD) > 18mm = sensitive, (MIZD) 14 – 18 mm = intermediate (MIZD) <14 mm = resistant, Concentration of extracts 100mg/ml

Table 5: Healing % of *Punica granatum* methanolic extract

Days	Control	Tetracycline 3%	Extract
0	0%	0%	0%
1	12%	19.4%	29%
2	34.6%	35.6%	50.8%
3	42.4%	39%	53.8%
4	49.4%	43.6%	60.4%
5	53.2%	49.6%	65.6%
6	58.4%	61.6%	70.6%
7	62%	65.6%	72%
8	68.6%	78.2%	78.8%
9	72%	82.2%	84.6%
10	77%	84.4%	93.8%
11	83%	92.2%	94.4%
12	87%	98.2%	97%
13	94%	100%	100%
14	97%	100%	100%
15	100%	100%	100%

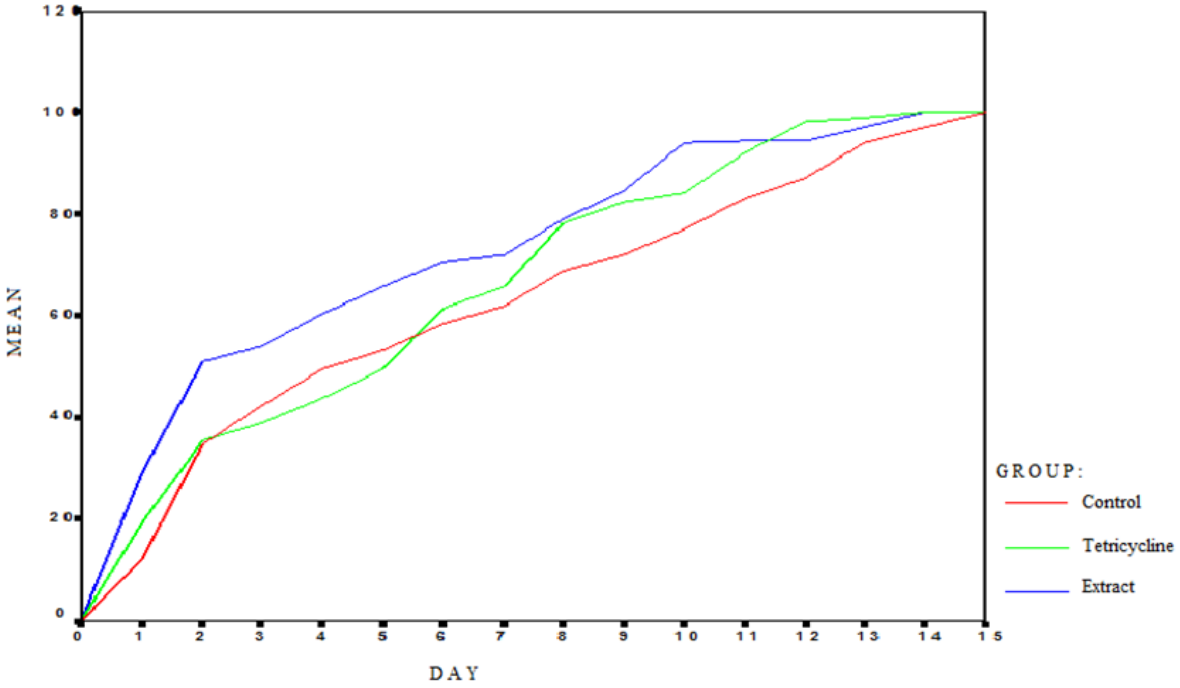

Figure 1: Healing % of *Punica granatum* methanolic extract

Ravindrasingh et al studied the effect of a topical *Punica granatum* peel aqueous extract on healing of burn wound and to compare its effect with that of standard (silver sulfadiazine).

The percentage of wound contraction was significantly increased in the topical Punicagranatum extract (10% and 20%) and silver sulfadiazine group compared to control group[16].

CONCLUSION

The results of this study indicated that *Punicagranatum*peel methanolic extract had high antibacterial activity and have good wound healing activity.

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