

Therapeutic effect of cold extract ethanol *Plantago Lanceolata* leaves ointment in induced infected burn-wound in mice

N. A. A. Al-Kafaji*, I. Kh. Kh. Hazza** and M. G. A. Al-Rubaie*

*College of Veterinary Medicine\ University of Baghdad

**Ministry of Science and Technology

Abstract

Medicinal plants have been reported to be very beneficial in wound-burn care, this study to evaluate the effect of topical application of *Plantago lanceolata* ointment comprising from dried cold alcoholic extract of *Plantago lanceolata* leaves 5 gm and 95gm of pure Vaseline on the healing of incisional wounds and burn contamination with 10^5 CFU/ml of *Staphylococcus aureus* and 1.5×10^8 CFU/ml of *Pseudomonas aeruginosa* in mice, this treatment divided in two steps, first step invitro experiment of extract effect on bacterial growth and second step invivo on dorsal back of 12 mice in two sites left and right back site which divided in two equal group (group A and group B) which subdivided in two groups (3 mice for burns -3 mice for wound) group A contamination with *Staph. aureus* and group B contamination with *Pseudomonas aeruginosa*, has been investigated the effect of *Plantago lanceolata* ointment on wound-burn healing were critically dependent on the time of application after wounding and burning, topical treatment starting at 2 day after wound- burn contamination, results showed significantly inhibition effect of *Plantago lanceolata* cold extract in growth of bacteria, so optimal effects on improving wound- burn healing were observed *Plantago lanceolata* ointment with concentration 5% significantly increased the rate and extent of wound- burn healing and inhibition growth of bacteria at 7-20 days after treatment compared with control site of wound- burn which contamination with bacteria without treatment where healing appearance in 26 days of wound- burn infection. This suggest that *Plantago lanceolata* ointment is effective improving wound- burn healing against contamination with bacterial infection as a back ground and it's important to investigate these herbal ointments activities without side and toxic effects. So short of treatment period treatment compared with classical case of wounds and burns.

التأثير العلاجي لمرهم أوراق لسان الحمل السناني المحضر من المستخلص الكحولي المبرد

في احداث الحروق والجروح في الفئران

نرجس عامر عبد الرزاق الخفاجي*، إيمان خلف خليل هزاع** وميثاق غالب عبد الربيعي*

*كلية الطب البيطري/ جامعة بغداد

**وزارة العلوم والتكنولوجيا

الخلاصة

تعد النباتات الطبية من أهم العلاجات الفعالة للعناية بالحروق والجروح، أعدت هذه الدراسة لتقييم تأثير استخدام العلاجات الموضعية لمرهم لسان الحمل السناني المتكون من 5 غم من المستخلص الكحولي المبرد الجاف و 95 غم من الفازلين النقي على التئام الحروق والجروح المستحدثة في الفئران والملوثة بجراثيم المكورات العنقودية الذهبية وبتركيز 10^5 وحدة تكوين مستعمرة/ مل وجراثيم الزوائف الزنجارية وبتركيز 1.5×10^8 وحدة تكوين مستعمرة، قسمت المعالجة إلى مرحلتين، الأولى شملت تجربة الزجاج وتأثير

المستخلص نمو البكتيريا، المرحلة الثانية داخل الجسم على المنطقة الوحشية الظهرية وعلى جانبيها الأيمن والأيسر لـ12 فارة والتي قسمت الى مجموعتين متساويتين مجموعة أ (6 فئران) ومجموعة ب (6 فئران)، ثم قسمت كل مجموعة الى مجموعتين فرعية (3 فئران لأحداث الحروق و3 فئران لأحداث الجروح) وتلويث المجموعة أ بجراثيم المكورات العنقودية الذهبية، وتلويث المجموعة بجراثيم الزوائف الزنجارية. يعتمد تأثير المرهم النباتي للسان الحمل السناني في التئام الحروق والجروح على وقت المعالجة بعد تلوث الحروق والجروح، بدأت المعالجة الموضعية بعد يومين من حدوث الإصابة. أظهرت النتائج التأثير الفعال للمستخلص الكحولي لنبات لسان الحمل السناني في تثبيط نمو الجراثيم في الزجاج، كذلك ظهر أفضل تأثير للمرهم عند التركيز 5% في زيادة معدل الألتئام في الحروق والجروح وتثبيط نمو الجراثيم. وقد ظهر أفضل تأثير للعلاج ما بين 7-20 يوم من تطبيق العلاج للمنطقة اليسرى مقارنة بمجموعة السيطرة المنطقة اليمنى غير المعالجة والتي أظهرت علامات الألتئام في اليوم 26 من الإصابة. أثبتت الدراسة فعالية المرهم المستخدم من المستخلص الكحولي المبرد للسان الحمل السناني في سرعة إلتئام الحروق والجروح بدون ظهور أي أعراض جانبية أو سمية. قصر فترة المعالجة مقارنة مع الحالات الطبيعية للحروق والجروح.

Introduction

Topical antimicrobial therapy is one of the most important methods of wound care and burns (1). Some medicinal plants have been employed in folk medicine for wound care (2,3). Some of these plants either possess pro-wounding-burns healing activities or exhibit antimicrobial and other related properties which are beneficial in over all wound-burn healing (4,5). Healing are conveniently classified into any of three types, healing by first intention, healing by second intention and healing by third intention, depending on the nature of healing by first intention are smoothly closed that no scare is left, wound healing by second intention are involve formation of granulation tissues which fill up the gaps between the wound edges are associated with significant loss of tissue, leaving little scare. Healing by third intention are usually those wounds left for three to five days until granulation bed falls before they are sutured resulting in extensive scars formation (6). Four distinct stages of wound healing have also been identified-inflammatory debridement, proliferation remodeling/ maturation stages. Wound healing processes are known to be influenced by among other factors by infections, nutritional status, drugs and hormones, type and sites of wound and wasting diseases like diabetes (7). In fokal are medicines, medicinal plants have been used widely in facilitating, wound healing with high degree of successes. This has inspired many researches which are aimed at a validating the claims and discovering mechanisms which possibly explains the potentials of these herbs on wound repair processes. In this study, we investigated ethanol extract of plantago lanceolata formulated in an ointment base for pro-healing activity on excision burns- wounds healing activity on burn- wound mice model. Plantagolanceolata which classified under plant family (Plantaginaceae) (8, 9), it contains flavonoids as luteolin and 6-hydroxyluteolin (10) β -hydroxyacteoside, 5-hydroxylated iridoids (11) 10-acetylaucubin, 10-O-acetylgeniposidic acid, phenolic compound such as tannins (12,13). The biological activities of plantago lanceolata leaves and seeds are wound healing, anti inflammatory, analgesic, antioxidant, antiulcerogenic (14) the extract of plantago lanceolata contains mixture of antioxidant may constitute one of the mechanisms that contribute to wound healing properties and stop bleeding (15).

Materials and Methods

Plant extract preparation: The plant leaves was collected in farm of veterinary medicine college at a period February to march then cleaned and speared out, dried in the laboratory at room temperature until they broken easily by blander, Two methods of extraction were employed first method (hot extraction): know amount of powder plant (100)g was extracted in soxhlet system used 500 ml of 70% of ethanol at 65 C° for 5 hour, the extraction was filtered through what man No.1 filter paper concentrated in rotary evaporator vacuum and then dried in electric oven 40 C° to obtain dry. The second method (cold extract): 100 g of powder plant leaves was put in 500 ml of pure ethanol in clean flask cover with slip-on then freezed at 20° for 9 days, extraction put in magnetic starrier for 30 minute than filtered, concentrated and dried in oven at 37°C for 3 days until use (16).

Preparation of culture media: Growth media prepare according to instructions are installed on the packaging for producing company: Pseudomonas media, staph 110, Muller Hinton, brain heart infusion broth, sterilized and placed in incubator at 37°C to ensure that no contamination then kept refrigerated 4°C until use.

Preparation of bacterial diluents dosage: (1, 1.5, 2, 2.5, 3)g of powder leaves extract was solvent in 10 ml of ethanol to prepare (100, 150, 200, 250, 300) mg/ ml to use in vitro experiment (17) ointment was prepare by using 5 g from plant extraction than mixed with 95 g of pure Vaseline, 0.1 pure glycerin until complete mixed keep it in clean container at 27-30 C° until use (18).

FT-IR diagnostic to plant extract and Chemical composition: The extract done in laboratory extraction/ Department of Materials Research/ the Ministry of Science and Technology-Baghdad, then examined samples by infrar-spectroscopy FT-IR spectro lab MB300. (diagram 1and 2). Chemical test: Depending on (19) method for detecting Glycosides, Saponoins, Flavonoids, Coumarins, pH, Tannins, alkaloids (Table 3).

Detection bioactivity test invitro: After detected the bacteria from laboratory of health ministry/ Al- Yarmok hospital and redetection from laboratory of zoonotic disease unite/ Collage of Veterinary Medicine, Baghdad University depended on (20). Use agare well diffusion method to measured inhibition effect of plantago lanceolata extraction on *Pseudo. aeruginosa*, *Staph.aueaus* growth prepared 1.5×10^8 cfu/ml from *Pseudo.arueagnosa* (21) and 10^5 cfu/ml from *Staph.aueaus* (22) as infected dose of bacteria.

Antibiotic sensitivity test: Depending on (23) method for bacteria sensitive test for 8 type of antibiotic that provide from India Hi media Company (Table 5).

Treatment experimental:

Animals: 12 white mouse (BALB/C) (25-30) gm in both sex were obtained from the animal house of the zoonotic disease unit of veterinary medicine, university of Baghdad and place in two equal group A and B groups, each mouse group were divided in to two sub group consist of mice (3 mice for burns and 3mice for wounds) the group A contaminated with *Pseudomonas aeruginosa* 1.5×10^8 CFU/ml (21) and group B contaminated with *Staphylococcus aureus* 1×10^5 CFU/ml (22).

Formulation of the ointment: Batches of the ointment were prepares and used in study. Batches contained varying concentration of extract 5 gm per 95 gm of ointment base (pure Vaseline), respectively to complete mixed with extract used glycerol (obtained from the formulation unit, department of pharmaceutics, university of Baghdad).

Wound-burn healing experiment: Shaving as seal of 1 cm diameter was impressed on the two site of dorsal back of mice depilated and sterilized with ethanol. Excision wound and hot plate to induced burns were inflicted according to methods described by (24) under light ether anesthesia, after complete we contamination wound-burn in right and left site of mice back with bacteria. Left sites were treated topically after 2 days of infection for (7-20) days to healing observed symptoms record result and take photo through treated period. The right sites (control site) were allowed to heal without treatment.

Results and Discussion

Herbal ointments containing different medicinal plants are used in folk practice to promote repair tissues. The medicinal plants that are so used owe their efficacy to a direct action on the wound repair processes or to the anti-inflammatory and anti microbial properties. In this study, topical application of ethanol cold extract of *plantago lanceolata* in to an ointment base on the excision wound-burn in mice back caused a significantly higher rate of wound healing and reduced the epithelilazation period to 14 days compared with burn which reduced epithililazation period to 18 days. Wound healing is a natural process of regenerating dermal and epidermal tissues. That agree with (25) which showed the wound healing, asset of overlapping events takes place to repair the damage in the inflammatory place, bacteria and debris are phagocytosed and removed and cytokines and mediators are released that cause the migration and division of cells involved in the proliferative phase. These evidence agreement with (26) showed angiogenesis, collagen depression, granulation tissue formation, epithelialization and wound contraction occur in the proliferative phase.

Results of FT-IR diagnostic to plant extraction and chemical contain: When examined the plant extract samples (hot and cold) method in FT-IR system the result showed different between cold and hot extract, cold extract method sample has more active group than hot extract method sample, that because of the colding lead to destroy pound between active group and increase the activity compound result agree with (16). Who explained that in some medical plant (Table 1 and 2) (Diagram 1 and 2).

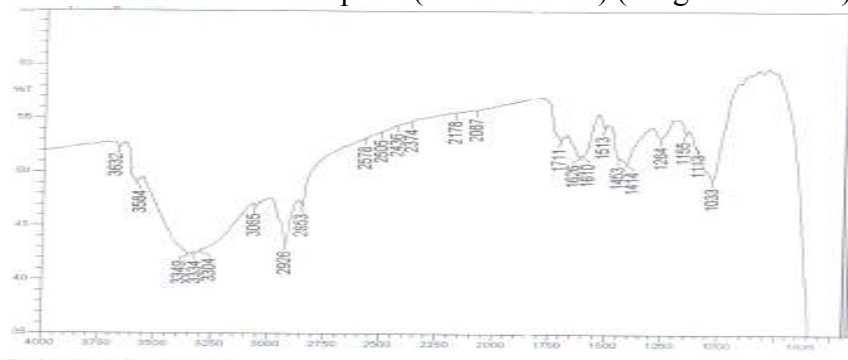


Diagram (1) cold method extraction to leaves *Plantago lanceolata* in FT-IR

Table (1) Result of cold method extraction to leaves *Plantago lanceolata*

No of compound	C-H	O-H	N-H	C=O	C-O	C-F
1	2853	3632	3304	1711	1155	1033
2	2926	-	3349	-	1264	-
3	306	-	-	-	-	-

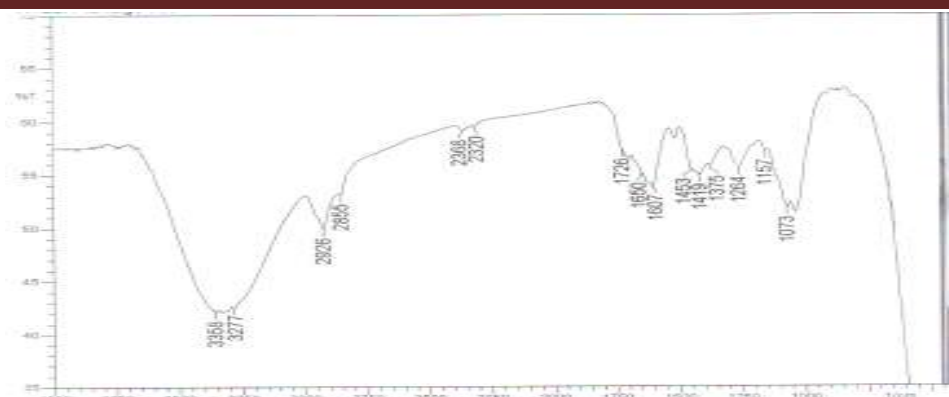


Diagram (2) Hot extraction method to leaves *plantago lanceolata* in FT-IR

Table (2) Result of hot extraction method to leaves *plantagolanceolata*

No of compound	C-H	O-H	N-H	C=O	C-O	C-F
1	2855	-	3277	1650	1157	-
2	-	-	3358	1726	1264	-
	-	-	-	-	-	-

Chemical contain of *plantago lanceolata* this study showed the chemical compound in this plant like coumarine, glycoside, saponnien, phenol and tainnin. This evidence was in agreement with (17) who explained that effect of this plant mastitis treatment of mice (Table 3).

Table (3) Result of biochemical test extraction of leaves *plantago.lanceolata*

Active group	pH	coumarin	Alkalioed	Glycoside	Saponnien	phenol	Tainnin
	5.8	+	-	+	+	+	+

Result of effect of extraction in vitro and antibiotic sensitive test: The result show that cold extraction of leaves *plantago lanceolata* mastery compared with hot extraction method give highest significant effect inhibitor zone in cold extraction than hot extraction (*staph.* 22.6 mm) and (*pseudo.* 21.6mm) at 300 mg/ml and lower inhibitor zone (*staph.* 17.5 mm) and (*pseudo.* 15.3 mm) at 100 mg/ ml while hot extraction give inhibitor zone (*staph.* 22 mm) and (*pseudo.* 21.3 mm) at 300mg/ ml and (*staph.* 13.6 mm) and (*pseudo.* 11.6 mm) at 100 mg/ ml due to full damage of plant cell wall and effect with freezing condition which allow to active compound to get down completely inside the extraction. This evidence was proved and established the observation reported by (16). 300 mg/ml of cold- hot extraction gives highest inhibition zone. Because pure ethanol solvent more amount of active compound this agree with (27,28). The result of screening plant extracts for antimicrobial activity was summarized in table (4) and figure:1 a and b) the result showed difference in inhibitor zone between two bacteria to the cold extraction that depending on nature of bacteria wall and have virulent factor *Staph.aureus* show high sensitive to extraction as compare with *Pseudo.eaagnosa* due to differ cell wall and repletion LPS (29) the result of sensitivity test agree with (30) which reported that *Staph.aureus* resist to 4 antibiotic: Trimethoprim, Tetracycline, Doxycyclin and Carbencilin; and sensitive to 4 antibiotic: Azethromycin, Gentamycin, Amoxicillin and Cefroxone while *Pseudo.aureagnosa* was more resistant to antibiotic that showed resistant to all types of antibiotic Table (5) summarized that. Also (31) reported that *Staph.aureus* have sever virulent that make it more resistance to most antibiotic, it can life inside lymphocyte and induce fibrin, abscesses that lead to form scar tissue prevent treatment to reach it, also converted to L-form when present

antibiotic and return to normal form when remove and produce enzymes that resistance to β -mithacillin, Lactimase resistance methicillin and pencillinase resist to penicillin compared with *Pseudo.aureaginos* which show more resistance to varies antibiotics (29). Recently, plant extract altered antibiotic that can prevent bacteria growth without side effect contrary chemical material that reduced from antibiotic which may hazareded on health (17) widely that plantago lanceolata has remarkable effects on bacterial growth, therefore this study focus on effect of Plantago lanceolata leaves as natural medical plant on pathogen causing typical (32).

Table (4) Result of effect of extraction invitro

Method of extraction	Concentration	Staph. aureus			Mean	SE	Pseudo. aerugenosa			Mean	SE
Cold extraction	A(100)	17	17.5	18	17.5	±0.2357	15	15	16	15.333	±0.3333
	B(150)	20	20	21	20.33333	±0.2722	18	17	18	17.667	±0.3333
	C(200)	21	21	22	21.33333	±0.2722	19	18	18	18.333	±0.3333
	D(250)	22	22.5	23	22.5	±0.2357	20	20.5	21	20.5	±0.2887
	E(300)	23	22	23	22.66667	±0.2722	22	21	22	21.667	±0.3333
	F(ZERO)	-	-	-			-	-	-		
Heat extraction	A(100)	13	14	14	13.66667	±0.2722	12	11	12	11.667	±0.3333
	B(150)	15	15	15.5	15.16667	±0.1361	16	16	17	16.333	±0.3333
	C(200)	18	18	19	18.33333	±0.2722	18	18	18	18	0
	D(250)	20	21	20	20.33333	±0.2722	20	21	22	21	±0.5774
	E(300)	22	22	23	22.33333	±0.2722	21	21	22	21.333	±0.3333
	F(ZERO)	-	-	-			-	-	-		

Results of some antibiotic disc:

Table (5) Result of sensitive disc test of antibiotic against bacteria

Sensitive disc	Concentration	<i>Staph.auerus</i>	<i>Pseudo.aeuginosa</i>
Azithromycin	Azm (15)	25 m	13 m
Trimethoprim	Tmp (5)	-	-
Gentamycin	CN	20 m	7 m
Amoxicillin+ Clavunin	Amc (30)	20 m	-
Tetracycline	T E	-	-
Carbencillin	Py	-	-
Ceftroxone	CRO	16 m	-
Doxycycline	Do	15 m	-
			-

Result and discussion of pathogenicity experimental: The wound is eventually closed by a combination of all these and by the process wound contraction. The wound is made smaller by action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cell. Collagen is remodeled and realigned along tension lines and cells that are no longer needed are removed by apoptosis. The plantago lanceolata based herbal ointment has significant influence on one or some of stages resulting in faster rate of wound- burn closer when compared to the un treated (right site) which showed healing after 21-26 days. Plantago lanceolata is widely distributed and has a wide range of folk application in the treatment of topical disease. This agreement with (17). She showed the effect of plant as a mastitis treatment. It's used remedy for applied externally to swelling, bruises and inflamed wounds (33). This plant has enzymes and anti-inflammatory activity in leaves, roots and seed has been demonstrated in rodent (17). Phytochemical analysis of ethanol extract used in formulating the herbal ointment showed the presence of most important constituent of the leaves are an iridoid, glycosides, saponins, tannins, mucilage,

flavonoid, carbohydrate, carotene, enzymes and silicic acid which have leaves astringent properties. This evidence agreement with (34) which showed the effect of plantago lanceolata of growth bacteria. Results show that experimental skin infection wound- burn that contaminated *Pseudo.aeruginosa*, *Staph. aureus* bacteria (high virulent infection) which cause redness, odema, suppuration of infected area with painfully when touch it and animal discomfort with diarrhea, the mice that infected with *Pseudo.aeruginosa* some animals died due to bacteria contain virulent factor like enzyme and entero toxin which help it to destroy and invasion tissue, redness and swelling of tissue because inflammatory reaction, accumulation of inflammatory cell and odema due to permeability of blood vessels which pressure on sensitive nerve cause pain and discomfort animal that result agree with (29, 35) suggest that *Pseudo.aeruginosa* more virulent effect compared with staph. aureus, causing diarrhea to animal due to bacteria have enterotoxin which speared throw digestive system (29) via exotoxin spread factor which destroyed tissue and cause septicemia this evidence was in agreement with (21) the bacteria adhesion occurs on epithelial cell surface (36) that happen in precision mechanism when bacteria produce protease which fibinonectin and destroyed epithelial cell throw virulent factor (phospholipase, rhaminolipids causing lipid broken down, lecithin in the cell wall that lead disorder of ionic and enzymes permeability inside and outside the cell that causing cellular necrosis and degeneration changes (37) that's agreement with (21).

Result and discussion of treatment experimental: Result showed gradually disappear of sever optical clinical singe like redness, swelling and diarrhea after 7 days of treatment with ointment of Plantagolanceolata leaves extract to mice infected, also there are no side effect appear after end of the treatment period. Because it contain components of therapeutic value act as anti inflammatory, wound healing like tannin, flavone and glycoside thes results were in acceptance with those previously reported by several investigator (14, 38) that reported the anti-inflammatory effect of *plantagolanceolata*. Also these extracts prepared from green plants that contained thiocyanate which act together with lactoperoxide in epithelium cells which act as antibacterial and stimulation of immunological system (37). That's agree with (39), found the important used of green plants as a food to limited of side effect and act as antidote this evidence was in agreement with (40). (41) reported that plant also contain vit A and Ca which help to bulid epithelial cell .this result agree with (42) which used the plant as antidote due to contain enzymes like acidic phosphatase, naphthol-As-biphosphohydrolase which increase healing in the damage tissue. The held examination showed that the application of green ointment at treatment of mentioned diseases causes the stimulation of regeneration processes, fast healing. The wounds are healed earlier than classic terms. At burn case there was absorbed the decrease and elimination of ache and plasmorrhhea. The treatment results incomplete epithalization without scars that's agree with (43).

The results of this work show that formulating plantago lanceolata extract in to an ointment is effective in wound repairs-burn healing and encourages the harnessing of the extracts in the formulation of commercial dermatological ointment. This study show at applying of green ointment there was no cases of prolongation and complication.

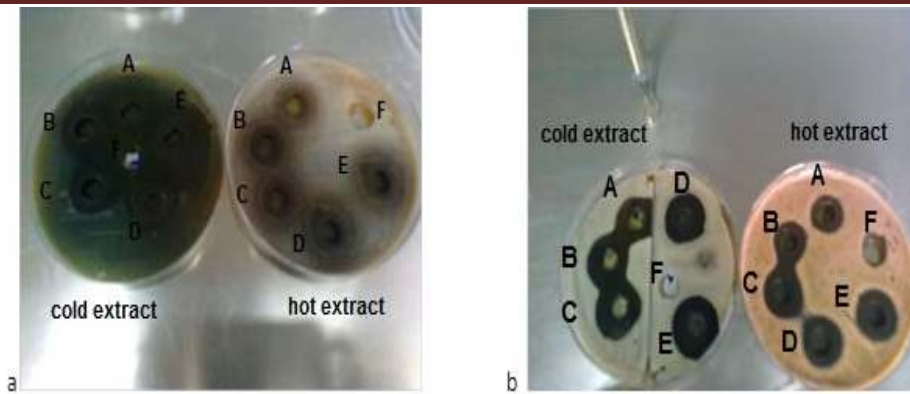


Figure 1: The inhibition effect of *plantago lanceolata* extract against *Pseudomonas aerogenosa* (a) and against *Staph. aureus* (b) invitro by cold extract (1), by hot extract (2).

A:100 mg/ml , B:150 mg/ml , C:200 mg/ml , D:250 mg/ml , E:300 mg/ml , F:alcohol (control).

Figure (2): Show (A) leaves of *plantago lanceolata* (Author, 2006) (B)Ointment of extract and (C)Solution of extract.

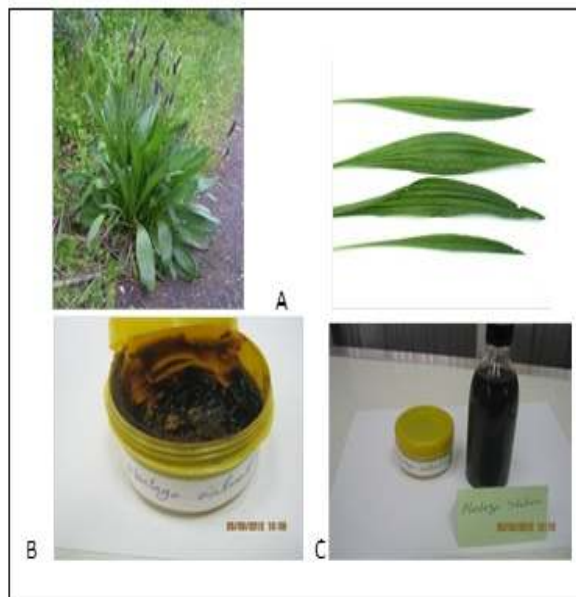


Fig. (3) Showing induce wound (A) and induce burn (B) which contamination with staphylococcus aures and wound (C) and burn (D) which contamination with Pseudomonas aerogenosa in back of mice.

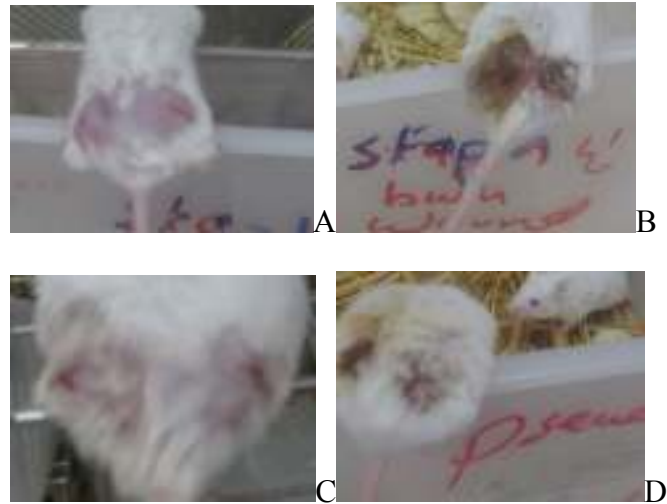


Fig. (4) Showing the left side of mice which treatment with ointment of Plantago lanceolata and the right said which is control effected with bacteria but not treated.



Fig. (5) Showing start of tissues healing in the left side which is infected and treatment with Plantago lanceolata ointment compared with right side (control), note (A) wound (B) burn contamination with staphylococcus aures, (C) wound and (D) burn which infected with Pseudomonas aerogenosa after 7 days of treatment .

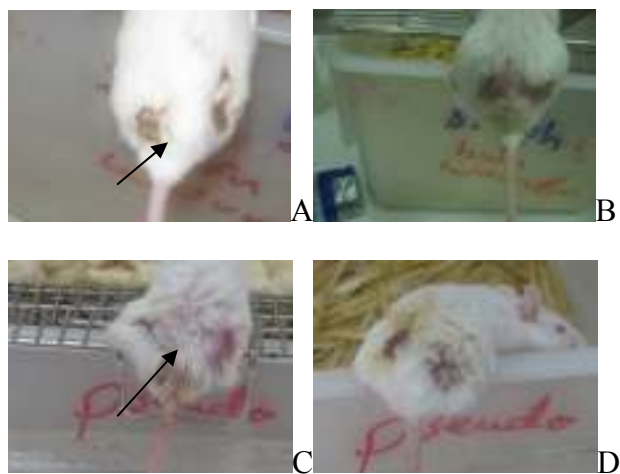


Fig. (6) Showing limited of infected area in the lift side of mice which treated with *Plantago lanceolata* ointment compared with right side which infected but not treatment wound (A) and burn (B) which infected with *staph. aurous*, (C) and(D) which infected with *Pseudo. aerogenosa* after 10 days of treatment.

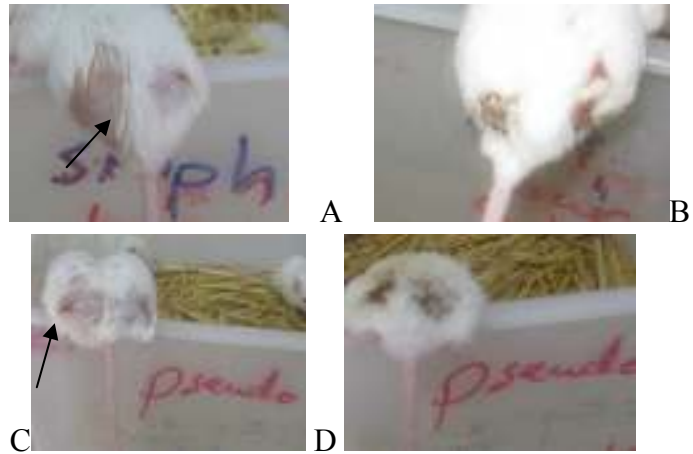


Fig. (7) Showing the clear red area of healing tissues compared with infected right area (without treatment). (A) Wound, (B) burn which infected with *Staph. aurous*, (C) wound and (D) burn which infected with *Pseudo.aerogenosa* after 14 days of treatment.

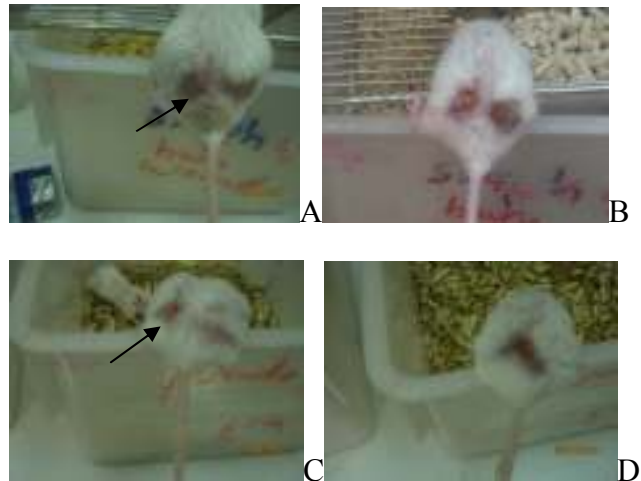
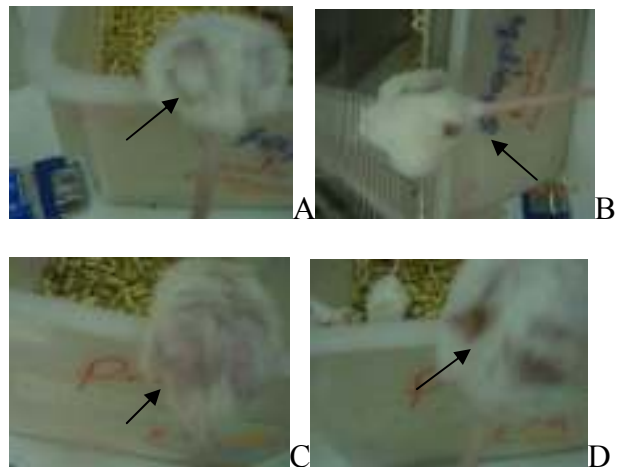


Fig. (8) Showing the complete healing, note healing of tissues and growth of hair in lift side (A) wound, (B) burn which infected with *Staph. aurous*, (C) wound and (D) burn which infected with *Pseudo.aerogenosa* compared with right side which infected without treatment after 20 days of treatment.



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