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ANTIMICROBIAL ACTIVITY OF SOME NATURAL PRODUCT AGAINSTINFECTED WOUND IN RAT MODEL WITH MDR

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ABSTRACT

Blood cultures used to detect microorganisms infecting blood. Bacteria may disseminate to blood stream leading to bacteremia which may developed to harmful consequences to body. 53 blood cultures were collected from pediatric cancer patients and investigated for their bacterial load. Most common bacterial isolates were *E. coli*, *MRSA* and *klebsiella pneumonia*. Pomegranate peel and miswak are natural products with various applications. Pomegranate peel and miswak extracts were tested against most common bacterial isolates and showing a very promising results. *In vivo* animal wound infection model were performed to compare effect of using traditional antibiotics alone or combined with pomegranatepeel or miswak. Macroscopic examination of skin was done to various treatments. Histological and physiological investigations for various testing groups reflecting the synergistic effect of natural products with antibiotics to enhance healing process. The present results highlight the possibility to use certain natural products for some beneficial uses after more verifications of results.

KEYWORDS: Bacteremia; Pomegranate peel; Miswak; Inflammatory markers; Oxidative stress.

INTRODUTION

Wounds are among the major and widely occurring pathologies. The wound healing process is essential to reform the damaged tissue and prevent its invasion by pathogens. Although

wound healing is a fundamental process, the infection of wound can cause significant delays within the repair and regeneration cycle (**Boussetta *et al.*, 2009**). It has been reported that a key factor in delayed chronic wound repair was the failure of the host response to combat multifactorial infections including Escherichia coli, Staphylococcus, haemolytic Streptococcus, Bacillus, Pseudomonas, and Proteus species. The understanding and control of the microbial infections are of greatimportance for the enhanced healing and management of wounds (**Parnell and Volk, 2019).** Continuous overuse of antibiotics has undoubtedly driven the development of their resistance. Inappropriate prescribing, extensive agricultural use, a decline in the availability of new antibiotics, and the various adaptations by which pathogenic bacteria obviate the effectsof antimicrobials have further increased the threat. As resistance to first- to fourth-generation antibiotics gains force, the development of new antimicrobial agents must be a priority if the problem is to be contained **(Akinduti *et al.*, 2022)**. The plants are a large and unexplored reservoir of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids which have shown antimicrobial properties **(Djouossi *et al.*, 2015).**

Most of pharmaceutical products have been discovered from natural extracts as well as molecules extracted from natural products (**Li and Vederas, 2009**). Recently, many research teams in pharmaceutical companies focus on investigation of synthetic molecules as well as application of advanced technologies including genetic engineering and artificial intelligence to explore effective agents from natural sources (**Harvey *et al.*, 2015**).

The pomegranate (*Punica granatum*) have anti-inflammatory, anti-oxidative activities on the signaling molecules in human colon cancer cell line. Pomegranate extract in different levels (5-60 mg/L) was efficient versus UVA- and UVB-induced alteration in SKU-1064 fibroblast cells of human (**Adams *et al.*, 2006; Zarfeshany *et al.*, 2014).** Pomegranate juice downregulate the action of serum angiotensin-converting factor and decreases systolic blood pressure. It causes reduction in carotid intima-media density, increases serum paraoxonase and reduces LDL level afterwards 1 year of using it. Thus it has beneficial effects in hypertension, hyperlipidemia, heart disease risk factors**,** hyperglycemia, fatty heart, obesity and insulin resistance **(Stowe, 2011).**

Many researchers have reported the anti-inflammatory action of pomegranate extract. through reduction of reactive-oxygen species (ROS) levels. Its major components are ellagitannins, ellagic acid and punicic acid. They metabolized by gut microbiota to yield urolithins which

have a role in NFκB activation and MAPK downregulation of COX-2 (**Boussetta *et al.*, 2009**).

Miswak contains a group of molecules and elements with a promising antibacterial activity especially cariogenic bacteria present in mouth. It has been *in vitro* tested either suspension or embedded in agar medium with very promising effects. It has selective action on *Streptococcus* mutants, *S. aureus*, Methicillin-resistant *Staphylococcus aureus* and *Streptococcus* species in comparison with chlorhexidine digluconate. *Salvadora persica* contains apigenin, luteolin, astragalin and kaempferol-3-*O*-rhamnoside, benzyl isothiocyanate with a promising action versus Gram-negative bacteria due to lipophilic and electrophilic properties, leading to penetration the outer surface of bacteria and may downregulate the bacterial redox system by alteration the membrane **(Al Bratty *et al.*, 2020).**

The present study is aimed at evaluating the antibacterial and therapeutic activities of the extract of *Punica granatum* and *Salvadora persica* against infected wound in the rat model and unraveling its mechanisms of antibacterial action.

MATERIALS AND METHODS

1. Preparation of natural products extraction

Miswak and pomegranate peel were purchased from various medicinal shops for further testing in the study.

500 gm powder of each natural product were soaked in cold water, hot water and ethanol 70% in suitable container, left at room temperature (37°C) for 24 hours, shake vigorously every 6 hours, total extracts were stored in freezer (-20°C) for future testing (**Huang *et al.*, 2021)**

2. Bacterial Isolates

Common Clinical identified bacterial isolates selected to be tested Escherichia *coli*, *Staphylococcus aureus* and Klebsiella pneumonia.Ethanol of pomegranate peels and mistwak extracts was assayed against Escherichia *coli*, *Staphylococcus aureus* and *Klebsiella pneumonia*

a. Mueller hinton agar preparation

Beef Extract 2.00 gm, Acid Hydrolysate Casein 17.50 gm, Starch 1.50 gm, Agar 17.00 gm, Distilled Water 1000 ml and adjust pH 7.3 ± 0.1 at 25ºC.

100 µl of various bacteria (107 CFU/ml) were spread onto the Mueller-Hinton agar plate. Various extracts (25, 50 and 60 mg) was put to filter paper discs and then put on the inoculated plated. After 24 h of incubation at 37°C, clear inhibition zones around the discs were measured and recorded. Vancomycin (30 µg) was used as a positive control **(Kamonwannasit *et al.*, 2013).**

3. Experimental animals

a. Animal model

One hundred thirty (130) Female Wister albino rats (12 weeks old) weighing 120–140 g were purchased from the animal unit of National research center, left for acclimation for ten days, and split into thirteen groups (ten rats each). Animals were feed with standard food (purchased from Egyptian Animal health research center) and water *ad libitum*. "Following laboratory animal care rules". Approval from the Institutional Animal Ethical Committee was taken prior to the experimental work.

b. Drug and Chemicals

Getamycin and Cirprofloxacin 0.3% (Epico, Egypt) oinments were used during the stepsof experiment

c. Wound model

Wounds were prepared in dorsal area of shaved animal's skin using concentrated sulfuric acid for 5 -10 seconds. After 12 h, dead tissues were removed using a sterile blade to produce superficial wound. All groups were treated same model. In this model, wound contraction and epithelialization period were monitored. A specimen sample from the healed wound tissue was collected from each rat for histopathological examination. Rats were sacrificed the end ofexperiment and blood was collected and processed to obtain serum for further experiments (**Parnell and Volk, 2019).**

4. Preparation of bacterial concentration

Various bacterial strains (MRSA & *Klebsiella pneumonia*) were grown in nutrient broth for 24°C for 24 hours. Using the improved secptomhotmeter method to detect bacterial count after growth in nutrient broth media relative to an optical density of bacterial solution to be used during the experiment **(Deshpande, Shonnard 2000)**.

5. Experimental design

One hundred and thirty male albino rats were divided into 13 groups as follows: -

Group (1): Animals skin were processed for wound preparation and act as control.

Group (2): Animals skin were processed for wound preparation and administration of 10^5 CFUof MRSA (Gram positive bacteria) over the wound.

Group (3): Animals skin were processed for wound preparation and administration of 10^5 CFUof *Klebsiella pneumonia* (Gram negative bacteria) over the wound.

Group (4): Animals skin were processed for wound preparation and administration of 10^5 CFUof MRSA over the wound, then administration of thin layer of Gentamicin ointment over the processed area twice daily for one week.

Group (5): Animals skin were processed for wound preparation and administration of 10^5 CFUof MRSA over the wound, then administration of thin layer of Ciprofloxacin ointment over the processed area twice daily for one week.

Group (6): Animals skin were processed for wound preparation and administration of 10^5 CFUof MRSA over the wound, then administration of thin layer of 50mg of miswak extract over the processed area twice daily for one week.

Group (5): Animals skin were processed for wound preparation and administration of 10^5 CFUof MRSA over the wound, then administration of thin layer of 60mg of pomgrante peel extract over the processed area twice daily for one week.

Group (8): Animals skin were processed for wound preparation and administration of 10^5 CFUof *Klebsiella pneumonia* over the wound, then administration of thin layer of 50mg of miswak extract over the processed area twice daily for one week.

Group (9): Animals skin were processed for wound preparation and administration of 10^5 CFUof *Klebsiella pneumonia* over the wound, then administration of thin layer of 60mg of pomegranate peel extract over the processed area twice daily for one week.

Group (10): Animals skin were processed for wound preparation and administration of 10^5 CFU of MRSA over the wound, then administration of thin layer of 50mg of miswak extract and Gentamincin over the processed area twice daily for one week.

Group (11): Animals skin were processed for wound preparation and administration of 10^5 CFU of MRSA over the wound, then administration of thin layer of 60mg of pomgrante peel extract and Gentamincin over the processed area twice daily for one week.

Group (12): Animals skin were processed for wound preparation and administration of 10^5 CFU of *Klebsiella pneumonia* over the wound, then administration of thin layer of 50mg of miswak extract and ciprofloxacin over the processed area twice daily for one week.

Group (13): Animals skin were processed for wound preparation and administration of 10^5 CFU of *Klebsiella pneumonia* over the wound, then administration of thin layer of 60mg of pomgrantepeel extract and ciprofloxacin over the processed area twice daily for one week.

After 21days, all rats were sacrificed under ether anthesia at fasting state, and the skin was fixed on formalin for histopathological and immunohistological investigation. Skin tissues were frozen for biochemical examinations (malondialdehyde (MDA) and supraxide (SOD)) and immunological analysis (TNF alpha and IL-1 beta).

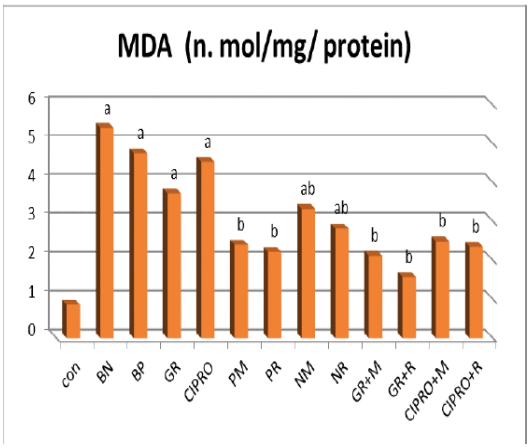
RESULTS

1. Physiologic testing

i. *Biochemical analysis (oxidative enzymes including (SOD and MDA)*

SOD level significantly decreased in the second infected groups relative to fist control group ($p < 0.05$), while upon treatment using either antibiotics alones or natural products of pomegranate peel and miswak SOD levels significantly increased ($p < 0.05$) as depicted in fig (10). It could be noted that maximal value of increasing of SOD levels could be detected upon using combinational therapy of both antibiotics and natural products in either Gram positive (*MRSA*) or Gram negative (*Klebsiella pneumonia*) wound infection model.

MDA level dramatically elevated in the second infected groups relative to fist control group ($p < 0.05$), while upon cure using either antibiotics alones or natural products of pomegranate peeland miswak MDA levels dramatically boosted ($p < 0.05$) as depicted in fig (10). It could be noted that maximal value of elevation of MDA levels could be seen upon using combinational therapy of both antibiotics and natural products in either Gram positive (*MRSA*) or Gramnegative (*Klebsiella pneumonia*) wound infection model (Fig.A).



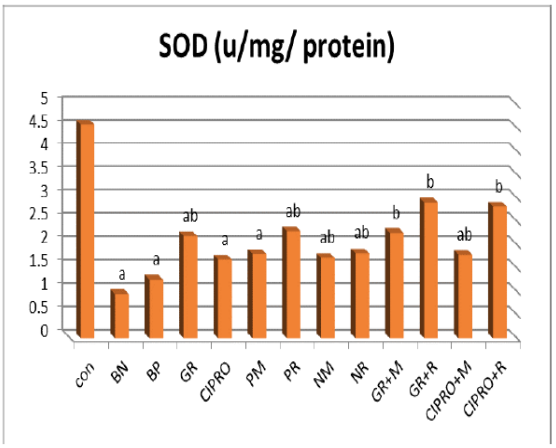


Fig (A): Oxidative enzymes including (MDA and SOD). (Data expressed as means ± SEM); a: statistically significant compared to corresponding value in control group (P<0.05); b: statistically significant compared to corresponding value in BN, BP groups (P < 0.05).

ii. Immunological studies (pro-inflammatory mediators including (TNF alpha and IL-1β):

Cytokines levels dramatically increased upon infection using Gram positive (*MRSA*) and Gram negative bacteria (*Klebsiella pneumonia*) relative to fist control group. A slight decrease inthe secreted cytokines upon administration of antibiotics alone (two types) as well as two extracts of pomegranate peel and miswak for the treatment of wound. It could be noticed that using combinational therapy of antibiotics with natural products respectively significantly decreased (cytokines levels in the last four groups ($p < 0.05$). These results reveled that using of combinational therapy improved protective cytokines effect leading to enhancing healing activityFig (B).

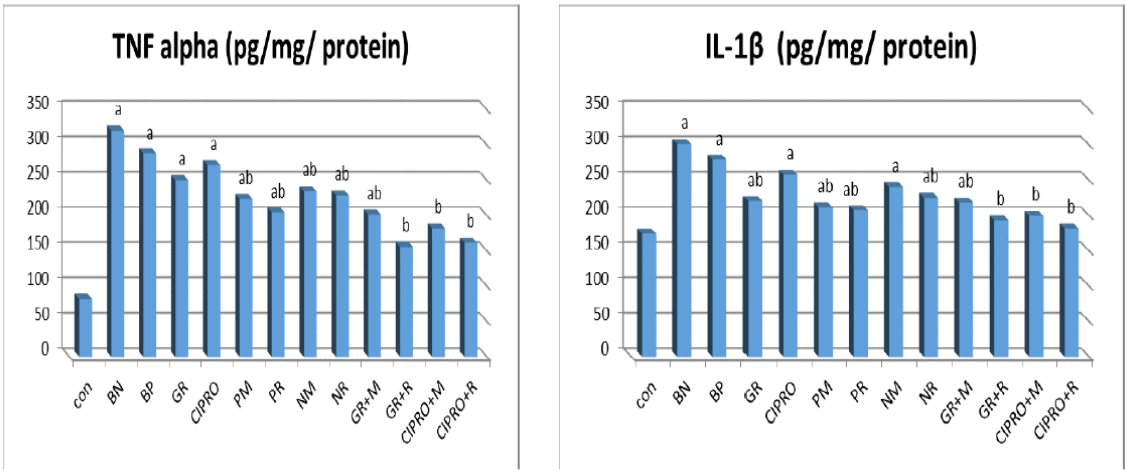


Fig (B): Pro-inflammatory mediators including (TNF alpha and IL-1β). (Data expressed as means ± SEM) a: statistically significant compared to corresponding value in control group (P<0.05); b: statistically significant compared to corresponding value in BN, BP groups (P <0.05).

Histopathological studies

Microscopic examination of hematoxylin eosin sections showed that: skin from the control group (**Fig. 1**) revealed normal structure of skin; the epidermis was made up of multiple cellular layers and rested on collagen rich dermal layer that contained hair follicles, sweat

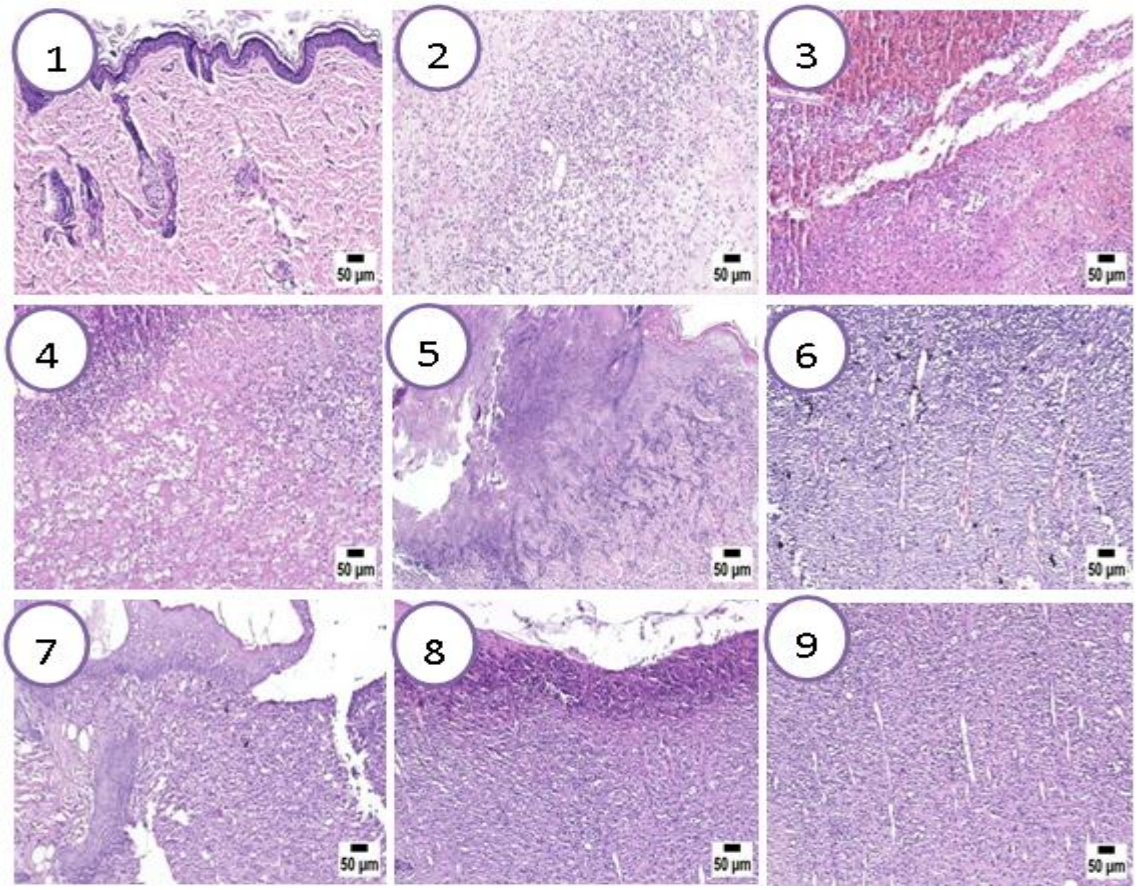
glands and sebaceous glands. The **BN, BP** group (**Fig. 2, 3**) showed signs of acute inflammation including intense neutrophils infiltration, marked edema and extensive hemorrhages. The wound gap contained enormous amounts of necrotic tissue debris.

Although showed thick cover of necrotic tissue debris with intense inflammatory reaction filling the wound gap. Granulation tissue formation was of minimal amount and was evident at the base of the wound (**Fig. 2, 3**).

Some of the examined wound sections from GR, CIPRO group (**Fig. 4-5**) were still hemorrhage with intense inflammatory edema and inflammatory cells infiltration. The content offibrovascular tissue filling the wound gap was minimal.

Wound area of F1 group (**Fig. 6-9**) showed mild improvement as the wound area contained granulation tissue that undergo organization toward the periphery with existence of intense inflammatory reaction.

Marked improvement was noticed in D group (**Fig. 10-13**) as the process of re- epithelization was started at the edges of the wound area with presence of hyperplastic epithelium.



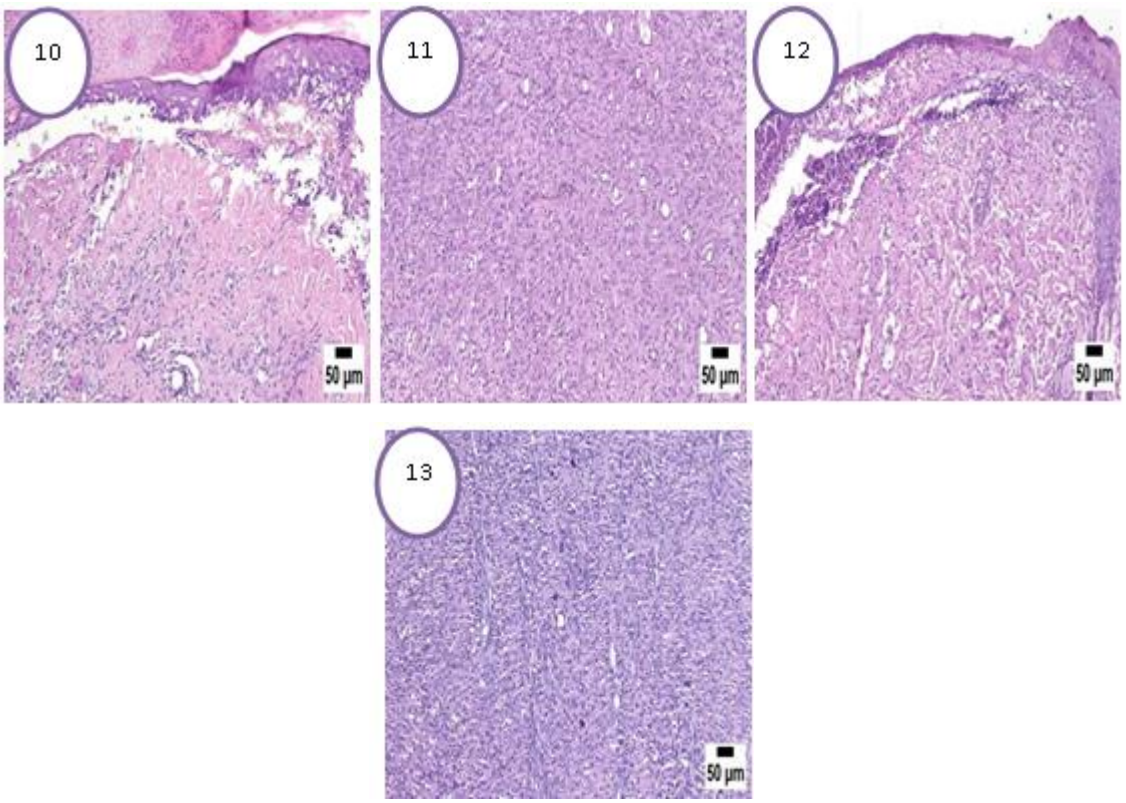


Fig. (1) Photomicrograph of skin, control group, higher magnification showing normal epidermis, dermis and skin associated structures. **Fig. (2)** Photomicrograph of skin, BP group, 21 day higher magnification showing intense inflammation at the wound area. **Fig. (3)** Photomicrograph of skin, BN group, after 21 days higher magnification showing extensive hemorrhage, necrotic tissue and intense inflammatory reaction at the wound area. **Fig. (4)** Photomicrograph of skin, GR group, after 10 days higher magnification showing amount of necrosis with intense inflammatory cells infiltration . **Fig. (5)** Photomicrograph of skin, CIPRO group, after 21 days higher magnification showing still amount of necrotic tissue with inflammatory cells infiltration . **Fig. (6)** Photomicrograph of skin, PM group, after 21 days higher magnification showing some congested blood vessels within the organized tissue, note marked inflammatory cells infiltration . **Fig. (7)** Photomicrograph of skin, PR group, after 21 days showing partial re-epithelization at the wound periphery with organized tissue filling the wound gap. **Fig. (8)** Photomicrograph of skin, NM group, after 21 days higher magnification showing enormous amounts of organized tissue filling the wound gap with necrotic tissue debris at the surface with inflammatory cells infiltration . **Fig. (9)** Photomicrograph of skin, NR group, after 21 days higher magnification organized tissue filling the wound gap with inflammatory cells infiltration. **Fig. (10)** Photomicrograph of skin, GR+M group, after 21 days higher magnification showing thick crust covering the wound surface with existence of

thin partial re-epithelization beneath . **Fig. (11)** Photomicrograph of skin, GR+R group, after 21 days higher magnification showing collagen rich fibrovascular tissue filling the wound. **Fig. (12)** Photomicrograph of skin, CIPRO+M group, after 21 days showing collagen rich organized tissue and improving fill the wound gap with thin epithelial cover. **Fig. (13)** Photomicrograph of skin, CIPRO+R group, after 21 days higher magnification showing collagen rich organized tissue filling the wound gap (H&E).

DISCUSSION

In the present study we used estimated doses of pommgrante peel as well as miswak extracts to be applied *in vivo* studies. It could be noticed that their MICs has been published in various research papers so we put an estimation depending on other groups published work **(Al-Ayed *et al.*, 2016; J EJ *et al.*, 2022)**

In the present study either miswak and pomegranate peel extracts were applied either alone or in combination with antibiotics to treat wound infected with most common Gram negative and Gram positive bacteria to assist in wound healing and prevent dissemination of microbes and it could be noticed that a combinational therapy have given the most successive results upon macroscopic examination of outer surface of skin reflecting the synergistic activity of miswak and pomegranate peel to eradicate bacterial infection and assisting in wound healing action.

It could be noticed that in order to address medical inequities, it is important to reconsider the use of natural items that are already ingrained in poor countries' cultures as medicines. Due to the limited membrane permeability, most substances are unable to reach their full potential due to low absorption and poor solubility. Due to a growing mistrust of contemporary medicine, conventional medicine has become more popular. As a result, studies need to be done on the protection, effectiveness, and optimization of herbal medicine (**Cary andPeterlin, 2018)**

Substances that encourage tissue differentiation and reproduction can speed up wound healing. Enhancing the proliferative capacity can hasten the healing of a wounds and relieve the participant's visual and physical suffering, even if it is rare for such a phase to be stopped or induce the formation of a wound infection. Reduced scar is also associated with enhanced cell growth; for example, fibroblast that proliferate more frequently tend to produce more collagen, which leads to a more formed wound site **(Ryall *et al.*, 2022).**

To reestablish the skin barrier function, the wound healing process involves a complex and crucially regulated series of various well-orchestrated molecular and physiological events. Inflammatory process, multiplication (, and maturity are the three distinct, but overlap, phases of healing process. The substances employed in the wound management can have a significant impact on wound closure and the effectiveness of healing process in occluding the wounded tissue. A wealth of knowledge on the function of conventional therapies in resolving the possible factors of unhealed injuries is available in numerous investigations. Conventional tissue repair remedies have been explored experimentally and practically.

In the present study it could be noticed that using combination therapy of pomegranate peel extract as well as miswak extract combined with antibiotics improve healing action for infected wound by *MRSA*, *E. coli* and *klebsiella pneumonia* after examining skin sections stainedwith hematoxylin eosin.

(El-Ashram *et al.*, 2021) reported that resources originating from plants, insects, and animals, as well as natural remedies based on nanomaterials and natural product-based nanoparticles to advance the future of wound healing. In particular, the therapy of wound healing has benefited greatly from the processing of new compounds derived from natural sources.

Inflammation, cell proliferation, and cell migration are the series of processes that lead to the repair of damaged skin, including the regenerate and restoration phases. Following damage, the following stage starts with vascular constriction, which promotes homeostasis and produces inflammation mediators. This event's primary purpose is to end blood supply to the wounds, not to restore injured tissue. Filling tissue development, mostly fueled by fibers and the angiogenic process, characterizes the proliferation stage. Myofibroblasts are produced at this step to cut the volume of the wound by vascularization and fibroblasts in the collagen fibers. Finally, collagen fibers are reformulated and improved along tension lines during the remodeling or maturity process to return the skin's typical firmness (**Bhubhanil *et al.*, 2021).**

Leukocytes are the first cells that extravasate and enter the site following injury and serve to start the cleaning of necrotic tissue cells as well as to fight off invasive bacteria. Some natural products could be applied to enhance leucocytes as Naoxintong, which includes 16 traditional Chinese herbal remedies. Major vascular disorders are often treated in clinics with Naoxintong. It is also applied in therapeutic settings for long-term diabetic problems. Prior to

theonset of hyperglycemia, Naoxintong therapy can address a number of processes connected to the disease, such as inflammatory cytokines, avoiding diabetes and its consequences. Using an injurymodel in Wistar rats, the extract of *Bergenia ciliata* demonstrated tissue repair activities (**Fang *etal.*, 2021).** In contrast to decreasing bacteria, wounds histopathology in the treatment groups showed enhanced collagenation, re-epithelialization, and neovascularization (**Kour *et al.*, 2021)**.

Monocytes and macrophages are regarded as essential components of the inflammatory phase of wound healing. By secreting matrix metallo-proteinases, these cells take role in the clearance of damaged matrix, cellular debris, and microorganisms. They also serve as a major supplier of chemokines that promote cellular proliferation and improve collagen biosynthesis. In line with numerous studies that demonstrated macrophages play a crucial role in the effective repair of skin wounds. Observed quicker contraction of the healed wound when licorice extract was topical application in comparison to untreated wounds or wounds that received the vehicle eucerin in rabbits. Because the production of protective matrix in fibroblasts and myofibroblasts causes cellular forces to be generated **(Assar *et al.*, 2021)**

(Kasouni *et al.*, 2021) demonstrated that the aqueous extract of *Urtica dioica* L. has the ability to heal wounds. By assessing the extract's impact on cell vitality, the cell growth, and chemotaxis, the capacity for healing process was assessed *in vitro*. To further understand the supporting pathways that effectively increase effect of wound *healing*, the anti-inflammatory and oxidative capabilities were then assessed. Additionally, a histological analysis of full-thickness injuries on rats was done in order to assess the wound healing capability.

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