WOUND HEALING (IN VIVO), ANTIOXIDANT, CYTOTOXICITY AND ANTI-MICROBIAL EFFECTS OF 3,4,5-TRIHYDROXY-N0-[(2-METHYL-1H-INDOL-3-YL)-METHYLIDENE] BENZOHYDRAZIDE (TIBH)

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Abstract - In new drugs development, hybrid molecules might possess synergistic result and thus a greater activity than each of its constituents. Subsequently 3,4,5-trihydroxy-N'-[(5-methyl-1H-indol-3-yl)-methylidene]benzohydrazide (TIBH) was previously synthesized upon the reaction of the appropriate indole carboxaldehydes with gallic hydrazide. In this study TIBH was tested for wound healing, antioxidant (DPPH and FRAP), cytotoxicity (MTT against human normal fibroblast) and anti-microbial activities. Minimal inhibitory concentration (MIC) method was performed to test TIBH antimicrobial effect against two strains of Staphylococcus aureus (ATCC25923) and Escherichia coli (ATCC 25922). Rats in Negative control, Positive control, TIBH low dose and high dose groups (n=6) were respectively topically treated with 0.2 mL of the vehicle (2% CMC /20% glycerol v/v), Intrasite gel, 10 and 20 mg/ml of TIBH once daily post wound induction surgery. After 15 days, wound closure percentage, histopathological changes of the wound sites (Hematoxylin and eosin and Masson's trichrome staining), Bax protein expression, SOD (superoxide dismutase) activity and malondialdehyde levels (representing lipid peroxidation) were evaluated and compared between control and TIBH treated rats. TIBH showed a remarkable DPPH radical scavenging activity, with IC_{50} values of 33.8 µg/ml, when compared to reference controls (ascorbic acid and quercetin). Cytotoxicity result showed that TIBH in concentration more than 154 µM (50 µg/ml) was toxic against human normal fibroblast with the IC_{50} equal to 77.4 µM (25.18 µg/ml). Antimicrobial assay reported that TIBH MIC against two tested strains was greater than the highest tested concentration of the TIBH (200 µg/ml). Evaluation of the effect of TIBH in wound healing showed that TIBH 10 mg/ml increased wound closure, promoted faster re-epithelization and collagen synthesis. It decreased number of inflammatory cells, decreased Bax protein expression and finally increased SOD activity and decreased lipid peroxidation. In conclusion TIBH showed to be a strong antioxidant and a wound healing promoter.

Index Terms - Wound Healing, Immunohistochemistry, DPPH, FRAP, Cytotoxicity, Antimicrobial.

I. INTRODUCTION

Physical, chemical, thermal, microbial, or immunological tissue trauma can disturb the tissue integrity and cause wound [1-3]. Wound healing is a multistep overlapping process includes hemostasis, inflammation, proliferation and remodeling. All phases must take place in proper order and time frame to heal the wound successfully. Wound healing involves various cells such as keratinocytes, fibroblasts and endothelial cells [4]. Hemostasis starts with vasoconstriction to stop bleeding. Bleeding on the site of injury will trigger platelets aggregation. Platelets will release clotting factors, growth factors and cytokines. These released factors lead the wound heling to its next step which is inflammation. During this phase the neutrophils go into the wound area to begin phagocytosis which eliminates debris and bacteria and damaged cells. Neutrophils are soon followed by macrophages. Macrophages continue cleaning of the wound and releasing of factors that progress the healing process. In the next step, proliferation, the wound area is filled up the granulation tissue. Fibroblasts begin to build new extracellular matrix and angiogenesis and reepithelization will occur. During the remodeling phase new collagen is produced. Many factors affect the wound healing like malnutrition, infection, insufficient oxygenation, stress, smoking, alcoholism's, age, diabetes and obesity [2].

Oxygen plays a substantial roles in wound healing process, including oxidative killing of bacteria, collagen synthesis, angiogenesis, and epithelialization. Both enzymatic and non-enzymatic antioxidants have a very important role in wound healing. The generated reactive oxygen species (ROS) attack occupying pathogens and destroy them. Though, extra production of superoxide harms the

adjacent tissues. Superoxide is dismutated to hydrogen peroxide (H2O2) and oxygen molecule (O2) by means of superoxide dismutase (SOD) or a spontaneous reaction to avoid the production of hydroxyl free radicals, the most detrimental ROS, [5]. Misuse of antibiotics to prevent bacterial infections resulted in increased bacterial resistance. The frequency of drug-resistant bacteria is increasing. Drug-resistant bacteria tend to spread epidemically in hospitals and are involved in producing nosocomial infections (Hospital-acquired infections). Gram positives Staphylococcus aureus is the highest abundant and Gram negatives Escherichia coli is among the highest abundant species were detected to infect the wounds [6, 7]

Wound and tissue linked diseases are the most known and damaging sorts of trauma around the world and thousands are still passing away each year due to the lack of adequate treatment. Failure of wound healing in a proper time and order results in chronic, non-healing wounds that need sustained supervisions and treatments. Over $25 billion is expected to be annually spent for the processes of chronic wound treatments only [8]. Wounds affect well-being, working capacity and independence of individuals [9]. Currently various techniques and strategies are being used for wound healing. In brief the approaches include the use of grafts, growth factors or siRNA, targeting microRNA, stem cell therapy, use of sensors to monitor and manage wound microenvironment, biocompatible and biodegradable wound dressings and use of marine-derived biological macromolecules, plant extracts and synthetic compounds [9-12]. Drug application on the skin has long been an excellent root due to its simplicity, availability of vast superficial space, substantial contact with the circulatory and lymphatic networks, and its non-invasive nature [13]. Due to the diversity of wounds nature, extensive range of wound dressings are nowadays available with new products target various phases of the wound healing procedure [14-19]. A perfect dressing should succeed in rapid healing. Despite recent efforts, wound therapy methods are often painful and inadequate which highlights the need of development of effective wound therapy methods, products and drugs (Uzun et al., 2013). Thus in this study the wound healing potential of a newly synthesized compound was evaluated as a wound dressing material.

Indolic compounds are biologically essential chemicals with various therapeutic potentials. They possess antibacterial, antifungal, antiviral, antimalarial, anti-HIV, anticancer, and antioxidant properties. They preserve lipids and proteins from peroxidation [20]. Gallic acid on the other hand is a polyphenol with various biological and pharmacological potentials. Polyphenols form a key class of chemo-preventive chemicals because they can inhibit ROS generation [21]. In the development of new drugs, hybrid molecules that might possess synergistic and more effective results compare to their constituents are of the great importance. 3,4,5-trihydroxy-N'-[(5-methyl-1H-indol-3-yl)-methylidene]benzohydrazide (TIBH) was synthesized upon the reaction of the appropriate indole carboxaldehydes with gallic hydrazide [20]. It is reported that the compound showed inhibitory effect on lipid peroxidation [20]. A previous study showed that in rat, TIBH pomote a dose dependent ulcer prevention potential against HCl/Ethanol-triggered gastric ulcer. It also revealed that TIBH is a safe compound in rats upon 2000 mg/kg oral administration [22]. In this study wound healing (in vivo), antioxidant, cytotoxic and anti-microbial activity of TIBH were evaluated.

II. MATERIALS AND METHODS

A. Materials

Intrasite gel (a trademark for Smith and Nephew Healthcare Limited), a colorless transparent aqueous gel, was used for local treatment of the positive control group wounds. It contains 2.3% of a modified carboxymethylcellulose polymer and propylene glycol (20%) as a humectants and preservative. Topical application of Intrasite gel on the wound absorbs the excess secretions and generates a moist condition at the wound without causing tissue maceration. It does not damage the surrounding skin or tissue and it is non-adherent [23].

The TIBH was synthesized by chemistry department, University of Malaya. Microanalyses were carried out on a Perkin-Elmer 2400 elemental analyzer. 1H-NMR and 13C-NMR spectra were determined with a Lambda JEOL 400 MHz FT-NMR (1H-NMR: 400 MHz and 13C-NMR: 100.4 MHz) spectrometer. Figure 1 illustrates the structural formula of the TIBH [20].

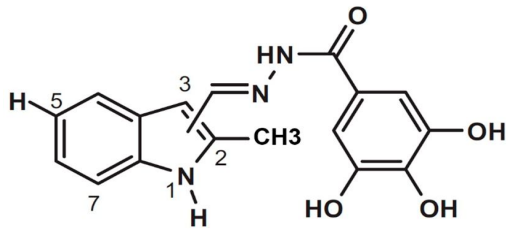


Figure 1. Structural formula of the 3,4,5-Trihydroxy-N 0-[(2-methyl-1H-indol-3-yl)-methylidene] benzohydrazide (TIBH) structural formula [20].

Dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany) is an organosulfur compound and an effective solvent dissolves both nonpolar and polar compounds. It is miscible in a broad range of organic solvents and water [24]. It was used to solubilize the TIBH.

Carboxymethyl cellulose (CMC) and Glycerol (Sigma Aldrich, Germany) were each separately used as a dressing vehicle [25, 26]. Here, combination of the two were used as TIBH vehicles respectively in concentration of 2% and 20%. Ketamine and xylazine were used to anesthetize and sacrifice of the animals (Sigma Aldrich, Germany). SOD and MDA commercial kits were purchased from the Cayman Chemical Company (Cayman, USA).

B. Evaluation of antioxidant activity; DPPH measurement

Free radical scavenging activity of TIBH various concentrations were measured by 1, 1-diphenyl-2-picryl hydrazyl (DPPH) assay as described by [27] with some modifications to be adjusted for 96-well plates. This method was theorized by [28]. Briefly, 0.1 mM solution of DPPH in ethanol was prepared. A total of 190 µl of DPPH solution (0.1 ml) was added to 10 µl of TIBH various concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 µg/ml) in DMSO. DMSO was used as blank. The plate was shaken vigorously and kept at room temperature for 30 min. DPPH solution has a deep violet color and its highest absorbance is at 517nm. The decrease in the reaction mixture absorbance specified the higher free radical scavenging activity and was measured at 517 nm by means of spectrophotometer. Quercetin and ascorbic acid were used as positive controls and each tests were done in triplicate. The concentration of TIBH required to inhibit 50% of the DPPH free radical (IC_{50}) was achieved using the TIBH dose dependent inhibition curve. The DPPH radical scavenging activity of each concentration was measured according to the following equation: DPPH scavenging effect (%) or Percent inhibition = [(blank absorbance − sample absorbance) / blank absorbance] × 100.

C. Evaluation of antioxidant activity; Ferric-Reducing Antioxidant Power (FRAP) measurement

This assay was carried out as reported by [29]. It was employed to measure the antioxidant potential of the compound for reducing colorless ferric complex (Fe^{3+}tripyridyltriazine) to a blue color ferrous complex (Fe^{2+}tripyridyltriazine) solution which absorbs the light maximum at 593nm. The FRAP reagent was freshly prepared by mixing 300 mmol/ L acetate buffer, pH 3.6, (prepared by dissolving 3.1 g sodium acetate trihydrate and 16 ml glacial acetic acid in up to 1.0 L distilled water) with 10 mmol/L TPTZ dissolved in 40 mmol/L of HCl solution and finally 20 mmol iron (III) chloride in distilled water solution ($FeCl_3.6H_2O$) in proportions of 10:1:1 (v/v), respectively. The FRP reagent was prepared fresh and was warmed to 37 C^o in a water bath before use. Quercetin and ascorbic acid were used as positive controls. TIBH, quercetin and ascorbic acid were serially diluted in DMSO from 100 µg/ml to 0.78125 µg/ml. A total of 10 µl of each sample was added to 300 µl of the FRAP reagent (final dilution 1: 34) in a 96 well plate. The absorbance at 593 nm was read in the beginning (At_0) and after 4 min (At_4) against a blank reagent (DMSO) using a spectrophotometer. The difference between the absorbance values was calculated for each sample. The blue color formation indicates that the sample has a reductant (antioxidant) potential. Known concentration of Fe^{+2}($FeSO_4.7H_2O$) aqueous solutions were freshly prepared and used for calibration. The concentration range over which the absorbance showed linear response (100– 1000 mmol/L) was used to calculate a standard curve. Results were expressed as mM of $FeSO_4.7H_2O$ / dry weight of the compound. All the tests were done in triplicate and the mean values were calculated [29, 30]

D. Human normal fibroblast cell (BJ-5ta) culture

Human normal fibroblast (BJ-5ta) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin (5000 units/mL) and streptomycin (5000 µg/mL). It is then incubated at 5% CO_2 at 37°C. Medium was changed every 2 days and cells were sub-cultured (split ratio 1:4) every 4 days by trypsinization (0.25% trypsin/0.02% EDTA) [31].

E. Cytotoxicity assay

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was used to evaluate the cytotoxicity of TIBH against human normal fibroblast (BJ-5ta) as described before with some modifications [32]. To obtain non-toxic concentration of DMSO for TIBH solubility, DMSO cytotoxicity against BJ-5ta fibroblast cells was tested using the same method. The reduction of tetrazolium salts is a broadly used efficient method to determine various substances cytotoxicity. The yellow tetrazolium MTT is reduced by alive cells, by the action of dehydrogenase enzymes to generate NADH and NADPH. Generated intracellular purple formazan can be solubilized and quantified by spectrophotometric. In brief, BJ-5ta cells were seeded into 96 plates at a density of 5×10^3 cells per well and incubated overnight. The cells then were treated with various concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 µg/ml of the TIBH in DMSO and media. Wells with same range of TIBH concentration without the cells were used as blank. Culture media alone was used as negative control. After 48 hours the wells content were discarded. MTT was dissolve in Dulbecco's Phosphate Buffered Saline, pH=7.4 (DPBS) to reach 5 mg/ml in concentration. A total of 10 µl of MTT Solution was add to each well to achieve a final concentration of 0.45 mg/ml. After 4 hours of incubation of the plate in the dark at 37^oC, the wells content were discarded and DMSO (as a MTT solvent)

was added to the wells. Then the absorbance was read at 590 nm. Graph of cell viability (%) vs. the compound concentration was plotted and the IC_{50} value of the TIBH against human normal fibroblast was determined.

F. Evaluation of antimicrobial activity

Bactericidal effect of TIBH was tested against two standard strains of Staphylococcus aureus (ATCC25923) and Escherichia coli (ATCC 25922) which are known pathogens to cause wound infections [33].The test was carried out at Department of Microbiology, Universiti Teknologi MARA (UiTM). TIBH was evaluated in seven concentrations of 200, 100, 50, 25, 15, 0 µg/ml using MIC (Minimal inhibitory concentration) method [34-36]. The MIC is defined as the lowest concentration of the antimicrobial agent that inhibits bacterial growth. Suspensions of equal to 0.5 McFarland standards (5×10^5cfu/ml) were prepared using pathogenic organisms. Sterile BHI Broth (Brain Heart Infusion Broth), TIBH stock solution of 1000 mg/ml in DMSO and bacterial suspension of 1×10^8cfu/ml in BIHB were mixed according to table 1 (test tubes 1-7). To check the sterility, BHI Broth alone was added to three separate test tubes (test tube 8). After overnight incubation at 37 C^o, tubes were observed for growth using DensiCHEK Plus (BIOMERIEUX, INC). The DensiCHEK Plus used to measure the turbidity of the microorganism suspension and to determine the McFarland value generated using a single wavelength of 580 nm. The McFarland value is proportional to microorganism concentrations and the reading range is 0.0 - 4.0 McFarland.

G. Experimental Animals

Sprague-Dawley (SD) rats have been generally used for wound model to evaluate the healing effect of various synthesized compounds or plant extracts because they are easy to handle and cost-effective comparing to other laboratory animals. A total of 24 adult female SD rats (8 weeks) weighing 250-350 g were purchased from the Animal Experimental Unit, Faculty of Medicine, University of Malaya. Rats for four groups of Negative control, Positive control, TIBH Low dose (LD) and High dose (HD) were randomly selected,(n=6/group),. Each rat was housed separately in cage for 3 days and they then grouped (3 in each cage). with (1 rat per cage). The animals were nourished with standard pellet and allowed access to tap water.

H. Experimental animal's ethic

Usage of animal in this test was approved by the ethics committee for Animal experiment of the Faculty of Medicine, University of Malaya, Malaysia (Ethic No. 2015-181201/BMS/R/MAAH) and was conducted according to the National Academy of Science's Guide for the Care and Use of Laboratory Animals [37, 38].

I. Induction of excisional wounds

The animals were anaesthetized with intramuscular injection of 50 mg/kg of ketamine (100 mg/mL) and 5 mg/kg of Xylazin (100 mg/mL) before wound formation. The skin was shaved and disinfected with 70% alcohol. A skin circle of 2 cm in diameter and 2 mm in depth was excised from the dorsal neck skin. Excisions in the tissues beneath the skin were prevented. The neck area of rat was chosen because it cannot reach it to bite or scratch [19].

J. Topical Application of the drugs

Rats in Negative control group were topically treated with 0.2 mL of the vehicle (2% CMC/20% glycerol v/v) once daily. Positive control group rats were treated once daily with 0.2 mL of Intrasite gel topically. Animals of TIBH LD and TIBH HD groups were treated once daily with 0.2 mL of the 10 or 20 mg/ml of TIBH in vehicle, respectively. The animals were daily monitored during the treatments [25].

K. Wound closure percentage

The required period to form a scar at wound area without any remaining raw wound is termed the epithelization period. Based on the former similar studies, this period takes 10 to 20 days [19, 39, 40]. In this study, wounds were photographed and wounds area were copied on transparent tracing paper respectively on day 0, 5, 10 and 15 of the experiment. The wound area was then measured using Image J (1.50i). The wound closure percentage (WC %) was calculated using the subsequent formula where WA (cm2) is the measured wound area [41]

(WC %) = [(WA at day 0 – WA at day X) / WA at day 0] × 100%

L. Histopathological Evaluation of Wound

On day 15, after euthanasia of the animals with overdose of 150 mg/kg of ketamine (100mg/mL) and 15 mg/kg of Xylazin (100 mg/mL) intramuscularly, the wound tissues with the surrounding normal skin were excised. Some of the tissues were fixed in 10% of phosphate buffered formalin and processed for histological assays and the rest were preserved for tissue homogenate. Fixed tissues were processed, paraffin embedded, sectioned and stained with haematoxylin & eosin and masson's trichrome. They were then examined under light microscope. Analysis of images has been performed by 2 independent experienced observers in a blinded fashion model for the treated and the control groups [42].

M. Immunostaining

Bax protein expression was evaluated in each formalin-fixed paraffin-embedded scar tissue section,

as previously described. The tissue sections were placed on poly-L-lysine coated slides The 5 µm skin tissue sections were washed with xylene to remove the paraffin. They were then rehydrated by graded ethanol solutions. Antigen recovery process was done using 10 mM of boiled sodium citrate buffer. Immunostaining was performed according to the manufacturer's protocol (DakoCytomation, Carpinteria, CA, USA). In brief, endogenous peroxidase activity was quenched using a peroxidase block. Scar sections were then incubated with Bax (1:200, Cat: ab7977; Abcam, Cambridge, MA, USA) biotinylated primary antibody for 15 minutes. The samples were softly washed with wash buffer followed by another 15 minutes of incubation with streptavidin–horseradish peroxidase. The sections were washed and then incubated with diaminobenzidinetetrahydrochloride (DAB) for 5 minutes and then counterstained with hematoxylin for 5 second. The samples were immersed 10 times in 0.037 M/L Ammonia and were washed with pure water earlier to the mounting of cover slips. The brown illustrations of tissue under a light microscope demonstrated the protein over expression.

Test tubes No.	TIBH in DMSO (1000mg/ml)	Bacterial suspension in BHI Broth (1×10^8 cfu/ml)	BHI Broth	Final concentration of TIBH	Final concentration of bacteria
Test tube 1	200 µl	5 µl	795 µl	200 µg/ml	5×10^5 cfu/ml
Test tube 2	100 µl	5 µl	895 µl	100 µg/ml	5×10^5 cfu/ml
Test tube 3	50 µl	5 µl	945 µl	50 µg/ml	5×10^5 cfu/ml
Test tube 4	25 µl	5 µl	970 µl	25 µg/ml	5×10^5 cfu/ml
Test tube 5	15 µl	5 µl	980 µl	15 µg/ml	5×10^5 cfu/ml
Test tube 6	5 µl	5 µl	990 µl	5 µg/ml	5×10^5 cfu/ml
Test tube 7 Growth control	0 µl	5 µl	995 µl	0 µg/ml	5×10^5 cfu/ml
Test tube 8 (Sterility control)	0 µl	0 µl	1000 µl	0 µg/ml	0 cfu/ml

Table 1. Preparation of TIBH various concentrations in bacterial inoculation of Staphylococcus aureus or Escherichia coli.

A. Tissue homogenization

Tissues were rinsed with ice-cold normal saline. The tissues were minced with scissors to the sections of 200 mg. One gram of minced skin tissue was mixed with 8 ml of phosphate-buffered saline (PBS). Tissue homogenization was performed using a homogenizer (Polytron, Heidolph RZR 1, Germany) with the addition of mammalian protease blockers. The tissue homogenate was centrifuged at 4,500 rpm for 15 min at 4°C [19].The supernatant was stored at -80°C for further evaluation of the lipid peroxidation (MDA) and SOD enzymatic activity using commercial kits.

B. Evaluation of enzymatic activities; Lipid peroxidation

Peroxidation and degradation of lipids happens due to the cellular injuries is an indicator of oxidative stress. Cayman's TBARS assay kit was used to detect the Malondialdehyde (MDA) (An end product of lipid peroxidation). A total of 100 µL supernatant of homogenized negative control, positive control and TIBH-treated group's tissues were separately added to 100 µL of SDS solution and 4 mL of the color reagent and then boiled (for 1 hour). After that the reaction was immediately ended by transferring the mixture into the ice bath (for 10 min). The mixture was centrifuged for 10 min at 1,600g at 4°C. A total of 150 µl of the mixture was added to the 96 well plate in triplicate and the absorbance was read at 532 nm. MDA standard curve was plotted and the MDA value of each sample were calculated from standard curve.

C. Evaluation of enzymatic activities; Superoxide Dismutase (SOD)

Activity of the superoxide dismutase (SOD) in the supernatant of all the scar tissue homogenates was measured using a Cayman's assay kit as stated by the kit's instructions. SOD samples plus with the SOD standard were set up in the assay plate. Briefly, 10 µL of assay standard and 10 µL of the supernatant of negative control, positive control and TIBH-treated group's tissue homogenate were separately mixed with 200µL of diluted radical detector in the wells. Then 20 µl of diluted xanthine oxidase was added to the all wells. After 30 min incubation on the shaker, the absorbance was measured at 440–460 nm.

D. Statistical Analysis

All results were recorded as mean ± S.E.M. The statistical analysis of differences between the groups were analyzed using Analysis of Variance (ANOVA) (SPSS ver.20) by Tukey's post hoc comparisons. A p-value less than 0.05 was recorded to be significant

III. RESULTS

A. Evaluation of antioxidant activity; DPPH measurement

TIBH showed a remarkable DPPH radical scavenging activity (Figure 2), with IC50 values of 33.8 µg/ml,

when compared to reference controls (ascorbic acid and quercetin) with IC50 values of 77.8 and 138 µg/ml, respectively. It means at any specific concentration, TIBH captured more DPPH formed free radicals and has a significantly higher antioxidant activity compared to ascorbic acid and quercetin. Coefficient p-values for all the regression line were significant.

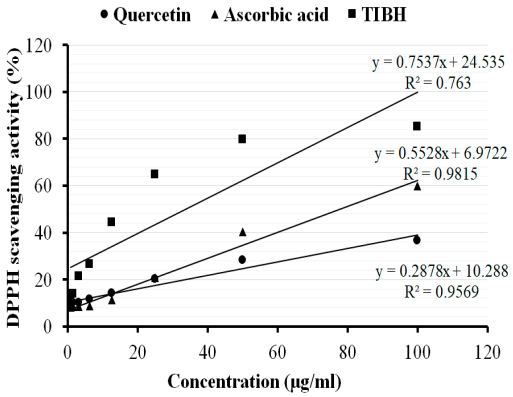


Figure 2 DPPH radical scavenging activity of various concentrations of TIBH, ascorbic acid and quercetin. Each value represents mean of the three replicates

B. Evaluation of antioxidant activity; Frap measurement

The reducing power estimated by the FRAP assay (Figure 3) displayed the TIBH offered more reducing power (p, 0.05) than the ascorbic acid and quercetin. The efficacy of the compound to transfer an electron to the TPTZ-Fe^{3+} is directly related to its antioxidant potential. According to the obtained results, the slope of the TIBH dose–dependent frap line was 20 times higher than the slope of ascorbic acid frap line and was almost same as the slope of quercetin frap line. So the compound sowed a very strong FRAP at the evaluated concentrations compared to positive controls. Coefficient p-values of FRAP regression line for ascorbic acid, quercetin and TIBH was significant.

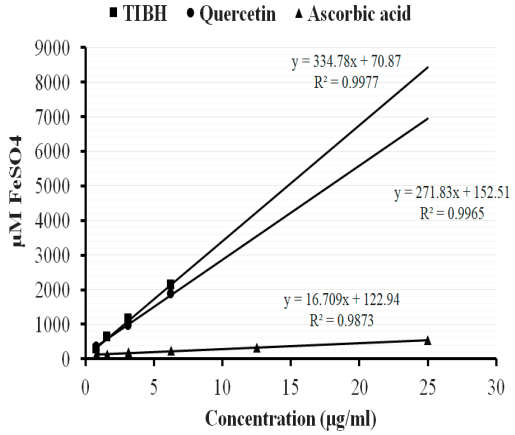


Figure 3. Dose–dependent frap plot of TIBH, ascorbic acid and quercetin over the concentration range of 0–100 µg/ml in the ferric reducing/antioxidant power (FRAP) assay. Each point represents the mean of the three readings.

C. Cytotoxicity assay

DMSO cytotoxicity assay against human normal fibroblast cells showed that DMSO was not toxic to the cells at concentration of 1% and below (Figure 4A). So DMSO in concertation less than 1% (V/V) was used to solve the TIBH. TIBH cytotoxicity assay result showed that TIBH in concentration less than 154 µM (50 µg/ml) was not toxic against human normal fibroblast and TIBH IC_{50} value for cytotoxicity was equal to 77.4 µM (25.18 µg/ml). Coefficient p-value of regression line of cell viability (%) versus various TIBH concentrations was significant (Figure 4B).

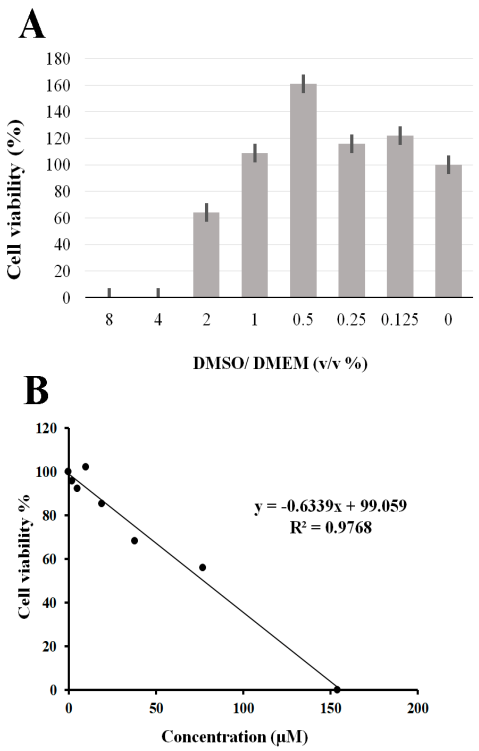


Figure 4. A) Cytotoxicity effect of the DMSO against human normal fibroblast cell lines (BJ-5ta). The result showed DMSO in concentration less than 1% in media was not toxic against human normal fibroblast. No cell growth was resulted at the 8% and 4% DMSO concentration. B) Cytotoxicity effect of the TIBH against human normal fibroblast cell lines (BJ-5ta). The result showed TIBH in concentration more than 154µM (50 µg/ml) was toxic against human normal fibroblast and the TIBH IC_{50} value obtained from the graph equals to 77.4 µM (25.18 µg/ml).

D. Antimicrobial Activity

All test tubes contained the inoculation of the bacteria showed the maximum McFarland value of 4 while the sterility control tubes had McFarland value of 0. The results indicated that there was no growth in the sterility control tubes (without bacterial inoculation) while the growth occurs in all bacterial suspension with various concentration of the TIBH. The obtained MIC for Chloramphenicol was 50µg/ml. TIBH MIC against two strains of gram positives Staphylococcus aureus and g negatives Escherichia coli was recorded greater than the highest tested concentration of the

TIBH (200 µg/ml).

E. Wound healing

Grossly, the wounds dressed with TIBH 10 mg/ml revealed a remarkable wound improvement and wound healing rate significantly accelerated in comparison with negative control group. As it is displayed in the figure 4 Intrasite gel treatment showed the highest efficacy for the wound treatment in comparison with other treatment. Topical dress of the wounds with 10 mg/ml of TIBH resulted in a wound-healing rate of equal to positive control group treatment. Topical dress of the wounds with 20 mg/ml of TIBH showed a moderate healing compared to vehicle alone dressed wounds (Figure 5). Wound closure was also measured among all groups and (Table 2) percentage of wound closure in the CMC/glycerol treated group (Negative control) was significantly lower when compared with TIBH low dose or Intrasite gel treated group.

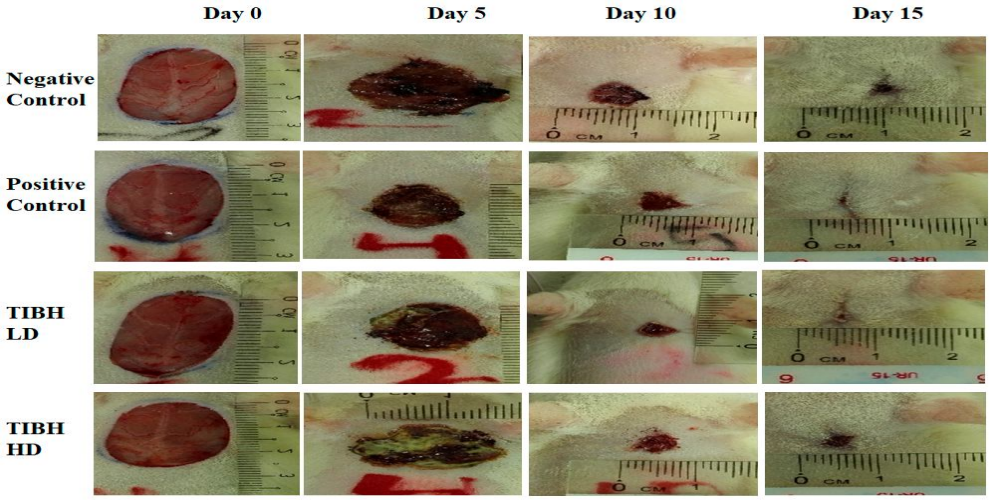


Figure 5. Effect of TIBH on the gross evaluation of wound healing on day 0, 5, 10 and 15.

A. Histopathological Evaluation of Wound

Figure 6A, 6B show histological wound analysis on day 15 after staining with hematoxylin and eosin that were observed respectively with 2X and 100X magnification. Figure 6C shows histological wound analysis of wound on day 15 after staining with Masson's trichrome which was observed with 2X.

B. Immunostaining

Immunohistochemistry staining of Bax protein on the other hand demonstrated that wounds treated with vehicle had an increase in the expression level of the Bax protein. Bax expression level decreased upon the TIBH administration of 10 mg/ml concentration and the results was comparable with intra¬site gel treatment (Figure 7)

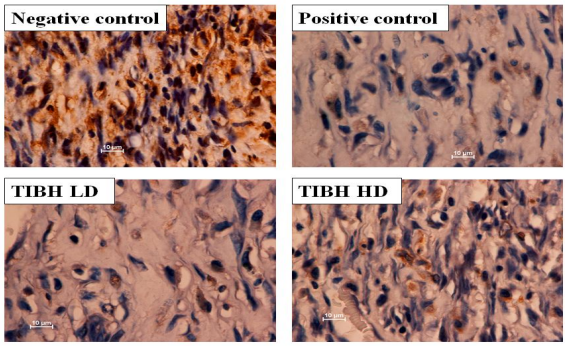


Figure 7. Immunohistochemical analysis of Bax protein in wounds tissue. Immunopositivity of Bax protein was represented in brown color staining.

C. Evaluation of Lipid peroxidation and SOD activity

Topical treatment of wounds with TIBH low dose and intrasite gel significantly reduced MDA level when compared to the vehicle alone treatment (negative control group) (Figure 8). This result suggests that TIBH treatment at low doses remarkably decreased the lipid peroxidation in the rats wound area. Results showed that topical treatment of rats wound with TIBH (10mg/ml) and intrasite gel significantly increased the activity of SOD when compared with the effect of vehicle treatment (Figure 9).

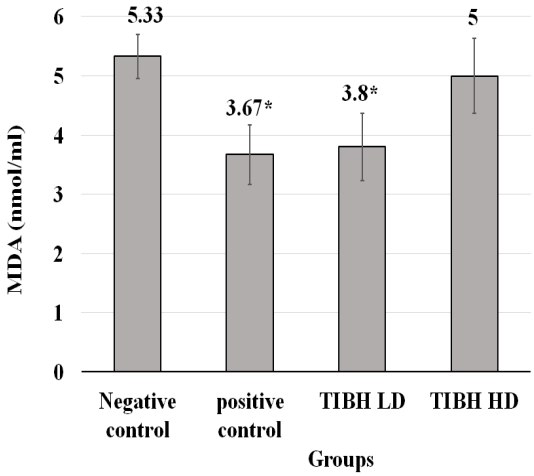


Figure 8. MDA level in the wound tissue of four groups of rats.

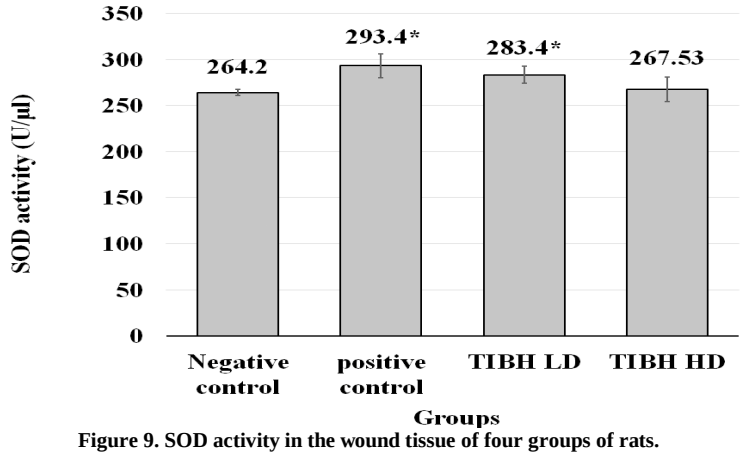
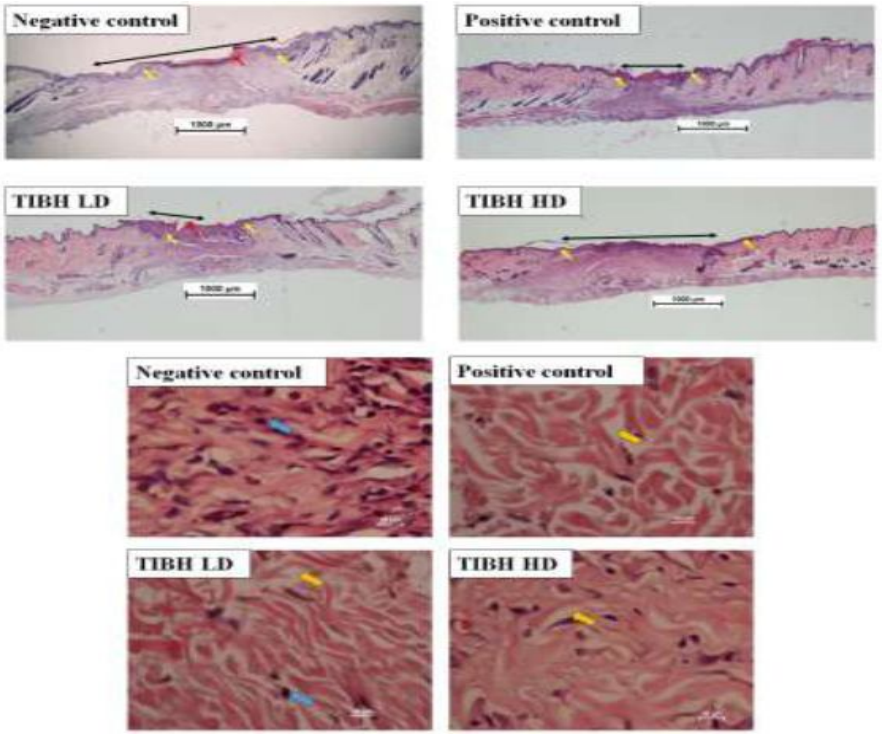


Figure 9. SOD activity in the wound tissue of four groups of rats.

	Day0	Day5		Day10		Day15	
Groups	Wound area (cm2)	Wound area (cm2)	Closure %	Wound area (cm2)	Closure %	Wound area (cm2)	Closure %
Negative Control	3.77 ± 0.03	2.24 ±0.07	40.64 ± 2.08	0.46 ± 0.05	87.89 ± 1.35	0.16 ± 0.02	95.88 ± 0.51
Positive Control	3.74 ± 0.15	1.49 ± 0.06*	60 ± 1.48*	0.24 ± 0.02*	93.7 ± 0.44*	0.04 ± 0.03*	98.91 ± 0.94*
TIBH LD (10 mg/ml)	3.67 ± 0.04	1.57 ± 0.03*	57.15 ± 0.6*	0.28 ± 0.01*	92.27 ± 0.35*	0.02 ± 0.0*	99.54 ± 0.30*
TIBH HD (20 mg/ml)	3.83 ± 0.19	1.88 ± 0.09*	49.97 ± 4.58*	0.44 ± 0.03	88.25 ± 1.25	0.08 ± 0.04	97.44 ± 0.91

Table 2. Effect of TIBH on wound closure percentage (%) in experimental rats
Negative control) 2% CMC / 20% glycerol v/v (vehicle); Positive control) Intrasite gel; TIBH LD) 10 mg/ml of TIBH was dissolved in vehicle; TIBH HD) 20 mg/ml of TIBH was dissolved in vehicle. Statistical analysis of the data was carried out using one way analysis of variance (ANOVA) and Tukey's post hoc test for average comparison on SPSS 20.0. Mean values ± SEM and n=6 animals in each group were used. Significance was defined as $*P< 0.05$ compared to negative control group.



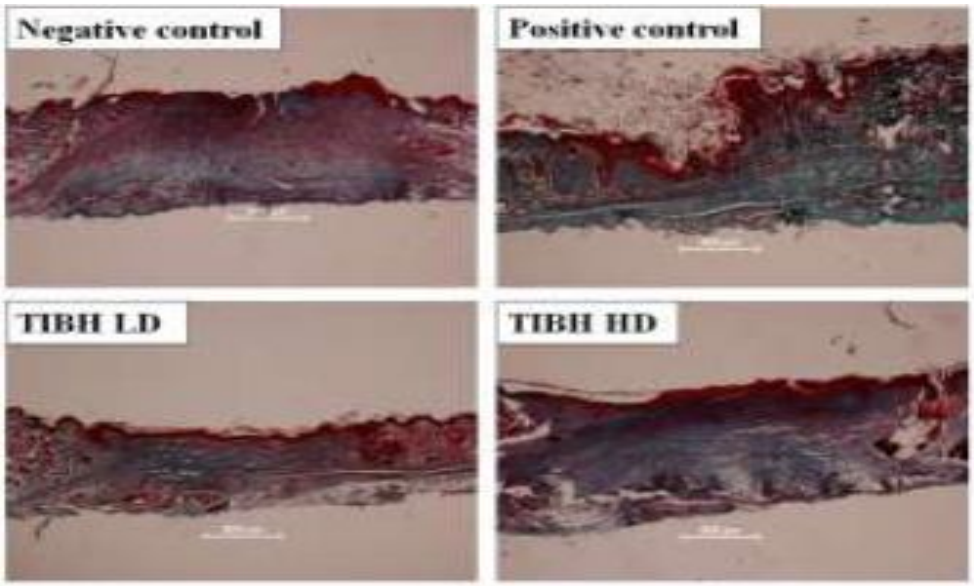


Figure 6. A) Histology analysis of wounds tissue stained with hematoxylin and eosin, 15 days after the wound induction (2X magnification). Negative control (2% CMC / 20% glycerol v/v) showed a wide wound area (black arrow); Positive control group (Intrasite gel) showed smaller wound area (black arrow) compared to negative control; TIBH LD (10 mg/ml) likewise showed a small wound area (black arrow), TIBH HD (20 mg/ml) showed a moderate wound area compared to negative control. Red arrow = scab, yellow arrow = epidermis. B) Histology analysis of wounds tissue stained with hematoxylin and eosin, 15 days after the wound induction (100X magnification). Negative control showed many inflammatory cells, least number of fibroblasts and little amount of collagen disposition; Positive control group showed remarkably more collagen fibers and fibroblasts with less inflammatory cells compared to negative control; TIBH LD group (10 mg/ml) showed noticeably less inflammatory cells and more fibroblast and collagen deposition; TIBH HD group (20 mg/ml) showed moderate number of inflammatory cells, a few number of fibroblasts and little amount of collagen disposition compared to negative control. Blue arrow = inflammatory cells, yellow arrow = collagen. C) Histology analysis of wounds tissue stained with Masson's trichrome, 15 days after the wound induction. Collagen fibers deposition in granulation tissues just below the wound site is observable in greenish blue color. Negative control group showed lesser amount of collagen deposition in granulation tissue of the wounds site compared to TIBH treated groups and Positive control.

DISCUSSION

It has been agreed that antioxidants play a substantial part in the maintenance of infected and non-infected wounds through the decrease of oxidative stress induced damages [13]. Previous studies also showed that antioxidant compounds assist to regulate wound oxidative stress and therefore speed up wound healing [43]. Therefore in this study, the improvement of rat skin's wound healing due to the topical administration of TIBH is suggested due to its strong antioxidant properties. Strong antioxidant activity of TIBH was proven by DPPH assay results. TIBH IC_{50} value (33.8 µg/ml) was nearly 2 times lower than ascorbic acid and 4 times lower than quercetin which are both known strong antioxidant chemicals. In this DPPH study ascorbic acid was used as a reference antioxidant and its IC_{50} value was recorded to be equal to 77.8 µg/ml. Some of the previously reported ascorbic acid DPPH IC_{50} values were such as 5.85 µg/ml [44], 16µg/ml [45], 50µg/ml [46], 62 µg/ml [47], 79.84 [48], 110.78 µg/ml [49] and 172.77 µg/ml [20]. The obtained DPPH IC_{50} value for ascorbic acid in this study was found to be similar to the reported IC_{50} values by [46], [47] and [48] and was informed to be higher than quercetin. DPPH assay is impressively affected by solvent impurities and changes of the pH. Therefore it is less robust than FRAP assay and many studies have used the FRAP assay for the general analysis of antioxidant activity of various materials and chemicals [29, 50-52]. In this study however, strong antioxidant potential of TIBH was also confirmed by the result of the FRAP assay. TIBH various concentrations possessed remarkably higher FRAP values in comparison with reference controls.

Fibroblasts are the most important cells in the process of wound healing. They are very important for angiogenesis, epithelialization and collagen formation initiation. In the progressive trauma healing, many of fibroblasts mature into myofibroblasts that promote wound closure [53]. This study showed TIBH in concentration less than 50 µg/ml was not toxic against human normal fibroblast as evaluated by MTT assay. Remarkably, this concentration is more than TIBH IC_{50} value for DPPH radical scavenging activity (33.8 µg/ml). TIBH IC_{50} value evaluated by MTT against Colon and Brest cancer cells were respectively reported to be 21.0 ±1.5 µM and 27.9±3.1 µM [20]. However in our study TIBH IC_{50} against human normal fibroblasts was shown to be 77.4 µM. This is greater than the mentioned cancer cell lines IC_{50} values. This could suggest that TIBH might have an anticancer property that needs more studies. DMSO is used to solubilize poorly soluble drugs [54] and here we used it as a solvent of TIBH. It was previously reported that DMSO at concentration of up to 10% did

not produce any significant alteration and cytotoxicity in Caco2/TC7 cells [54] while a later report showed that DMSO in concentrations of 0.5% to 6% and above results in a dose-dependent damaging effects on cochlear organotypic cultured cells [55]. The result of our study showed that DMSO in concentration less than 1% in media was not toxic against human normal fibroblast which was comparable with [55] result. Therefore, in this study to minimize the interference effect of DMSO with TIBH in cytotoxicity assay, DMSO concentration was kept below 1%.

Macroscopic evaluation of wounds determined that topical application of TIBH (10mg/ml) on wound areas significantly improved the rate of the wound closure. This result was similar to the wound healing effect of some previously studied synthetic compounds and plant extras [39, 42, 56].

Formation of a new epithelial layer is one of the most central procedures of the wound healing. Re-epithelialization happens when keratinocytes start to divide and occupy the wound surface [57]. Histological analysis showed that TIBH treatment decreased the wound width and increased the re-epithelialization. On the other hand collagen as a major component of mammal proteins and the most abundant factor of granulation plays a substantial role in all phases of wound healing and it acts as a structural scaffold in tissues beside its recently discovered substantial functional roles [58]. Histological analysis of TIBH revealed that the wound healing potential of TIBH might be due to its ability to increase the number of the fibroblasts, the amount of collagen deposition and to decrease the number of the inflammatory cells. Similarly other studies has reported that skin wound healing improvement increases with the increment of the collagen deposition and the decrement of the number of the inflammatory cells [25, 59-61].

Apoptosis reported to occur due to the expressions imbalance of BCL-2 family anti-apoptotic proteins and apoptotic Bax proteins [62, 63]. Bax expression increases markedly with the increment of apoptosis and it shows a strong association with the apoptosis in the process of wound healing [64]. Dysregulation in apoptosis can result in wound healing complications such as hypertrophic scar and keloid [65]. This study also showed that TIBH wound healing potential might be due to its effect on Bax Down-regulation as previously reported about other wound healer [3, 19].

Superoxide anions are the major ROS formed from molecular oxygen and they are rapidly detoxified by the action of SOD. In this study, local treatment of the wound with TIBH low dose significantly increased the SOD activity. Previous studies showed that SOD promotes the wound healing. SOD mRNA was shown to be overexpressed in wound area as verified by an RNA protection assay and in situ hybridization [5, 66]. It has been also reported that a single regimen of a cutaneous gene therapy for SOD decreases the delay of healing of diabetic rats wounds [67]. Our study showed that increase of the SOD activity might be one of the TIBH adopted strategy to enhance the wound healing. Similarly, various former studied compounds and plant extracts were reported to improve the wound healing by the increase of SOD activity [19, 39].

Lipid peroxidation forms ROS and delays wound healing [68]. As a result, decrease of the lipid peroxidation could be a suggested mechanism in wound healing. A previous in vitro study showed that TIBH has an inhibitory effect on lipid peroxidation [20] our results also indicated that TIBH wound healing potential might be due to its ability to decrease the lipid peroxidation. In previous investigations on other chemicals, decrease of lipid peroxidation was suggested as a wound healing mechanism [19, 39, 68].

DPPH and FRAP assay results showed that TIBH has antioxidant activity. Enzymatic antioxidant potential of TIBH was also showed in SOD and MDA assays. Putting together, both enzymatic and non-enzymatic antioxidants properties of TIBH highlight it as a wound healer [69].

CONCLUSION

In conclusion it can be said that TIBH treatment improved the wound healing by the increase of the fibroblast accumulation, collagen deposition, endogenous antioxidant enzymes activity (SOD), and the decrease of the number of inflammatory cells, lipid peroxidation (MDA) and Bax expression. Cytotoxicity assay revealed that TIBH concentration more than 50 µg/ml was toxic against human normal fibroblast with an IC_{50} value of 25.18 µg/ml.

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