HSP70 AND INOS BIOMARKERS IN EVALUATING THE HEALING PROPERTIES OF *RUBIA TINCTORUM*

Ali Alsarhan

Department of Clinical Science, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Johor, Malaysia.

Kamal Mansi

Department of biological sciences, Faculty of Science, AL Al-Bayt University, Mafraq, Jordan

Talal Aburjai

Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan, Amman, Jordan

Ahed Al-Khatib

Forensic medicine and toxicology dept-faculty of medicine-Jordan, University of science and technology-Jordan

Muhammed Alzweiri

Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan, Amman, Jordan

Naznin Sultana

Department of Clinical Science, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, Johor, Malaysia.

Abstract

Introduction*: Rubia tinctorum* L. (F. Rubiaceae) has historical role in promotion of healing process of thermal injuries. The cellular changes associated with burn healing process mediated by the plant were investigated.

Methods and Results: Treated rabbits with dried hexane extract exhibited increase in percentage of burn contraction as compared to control. The data showed that the expression of HSP70 was higher in treated groups with the plant extract than control groups. On other hand the expression of iNOS was higher in control groups than treated ones. It was also

found that the plant extract possesses antimicrobial effect. It produced wider zones of inhibition for *S. aureus* and *B. substilis* when compared to the zones of inhibition of gram-negative isolates. And also MIC and MBC for the gram-positive bacteria were significantly lower ($p < 0.05$) than those of gram-negative bacteria.

Conclusion: It was concluded that the positive outcomes for healing from burns is due to the over expression of HSP70, low production of iNOS and also to the antimicrobial effect of the extract.

Keywords: Rubia tinctorum, HSP70, iNOS, wound healing, Biomarkers

Introduction

Burns are considered as a major causality factor of disability and death of subjects with age less than 40. The data show that the number of injured individuals who are required medical intervention in developed countries exceeding 2 millions annually (Levy and Moskowitz, 1982).

Heat necrosis resulted from injury due to thermal burns depends on the conductance of involved tissue (Lin et al., 2011). The thermal conductance of the burnt tissue relies on several factors: including, thickness of the involved skin (Ipaktchi and Vogt, 2009), degree of its pigmentation (Ipaktchi and Vogt, 2009), content of water of the skin and its blood circulation. The latter is considered as a major factor in determining the degree of burn (Jonkam et al., 2007; Ipaktchi and Vogt, 2009).

Herbal plants have historical role in promotion of healing process of thermal injuries as enhances coagulation, inflammation, fibroplasia, collagenation and epithelization processes (Ulubelen et al., 1995; Zhao et al., 2011; Kale et al., 2012).

In the present study, the cellular changes associated with burn healing process mediated by R. tinctorum were investigated. It was hypothesized that R. tinctorum facilitates burn healing process by inducing the gene expression of heat shock protein (HSP) and reducing the expression of inducible nitric oxide synthase (iNOS) as healing biomarkers of skin tissue of burnt animals.

It has been reported that HSP70 protein is the most highly induced target of heat shock and the best characterized HSP (Vandenbroeck and Billiau, 1998; Pockley, 2002; Pockley et al., 2002). HSP70 expression is a sensitive and accurate biomarker for evaluating the tissue regeneration rate. The rate of HSP70 up-regulation is directly related to the contact temperature and time. If tissue temperature elevates more than 5oC, substantial induction of HSP70 expression will be measured. Also the rate of HSP70 expression varies depending on

tissue and the cell type (Wilmink et al., 2006). Furthermore, the rate of HSP70 expression increases in response to thermal stimuli until certain threshold point, pursued with a decrease in the protein expression rate. The maximum of expression is normally happened after 12 hour from the exposure to thermal stress (Diller, 2006; Wilmink et al., 2007).

Nitric oxide (NO) is also considered as a biomarker of cell regeneration rate which can be used to evaluate the wound healing process (Jezek and Watson, 2006; Tan et al., 2006; Yu and Bian, 2011). Inducible NOS (iNOS) is not usually active under normal conditions, it is activated due to the effect of proinflammatory agents in tissue suffering from thermal trauma (Lu and Cooper, 2011; Xie and Xue, 2011).

Materials and methods

Chemical and materials

All chemicals were purchased from Sigma-Aldrich, US except vaseline which was purchased from medical scientific and chemicals, Jordan, flamazine (silver sulfadiazine, Smith & Nephew, UK), paraffin wax (Paraplast, Lancer, and USA, Melting point = 53oC) and iNOS and HSP70 kit (Santa Cruiz, US).

Plant material and preparation

Three kilograms of *Rubia tinctorum* L. (F. Rubiaceae) were collected from the local region in North Badia. The plant was taxonomically identified by direct comparison with authenticated sample of the herbarium of faculty of sciences, department of biology sciences and with help of Prof. Al-Eisawi, D.M. Roots were separated, dried and finally powdered to be used in this experiment. Three formulas were prepared; formula A contains 10g of dried hexane extract of the plant, 80g vaseline, 5g beeswax and 5g olive oil extra virgin. Formula B contains 20g of the dried extract, 70g vaseline, 5g beeswax and 5g olive oil extra virgin. Formula C, the negative control, contains 80g vaseline, 10g beeswax and 10g olive oil extra virgin. Flamazine was used as a positive control.

Experimental animals

Twenty adult's female New Zealand rabbits (with average weight of 1300 g) were obtained from the central animal house of the medical college at Jordanian University of Science and Technology and were used in this study. The animals were kept ad libitum under standard laboratory conditions and veterinary supervision. The experiment complied with guidelines of the university of Jordan animal ethics committee which was established in accordance with the internationally accepted principles for laboratory animal use and care.

Burn induction

Burn induction was performed according to the "Protocol of Animal Use and Care at UC Davis" conforming to standard set by U.S. National Institutes of Health Bethesda, MD,

USA. Experimental burns should ideally be identical in depth and extent. A standard method requires exactly defining the size and location of the burn, the temperature gradient, the duration of exposure and the method of applying the burn. Standard burns were performed by techniques and device described by walker (Walker and Mason, 1968). Body surface was calculated according to Mech's formula:

$$A = 10W^{2/3} \quad \text{..... Eq.1.}$$

Where: A is area in cm2 and W is weight in grams.

Based on an average animal weight (1300 ± 12.7 g) and according to Mech's formula, the average whole skin area was 1190 cm2. 5% of total skin area (ca 60 cm2) was exposed to burn induction. The rabbits were anesthetized with 3.5% chloralhydrate solution, at a dose of 0.35 mg/g body weight intraperitoneally which is sufficient to prevent any movement of the animals at least for 2 h.

Hair was removed by shaving the dorsal back of the rabbits. Ethanol (70%) was used as antiseptic for the shaved region before burn induction. Burns were produced by immersing the rabbit's dorsal back in boiling water (99oC) for 10 sec resulting third degree burn. The burn was approximately 5% of the body surface.

Treatment of Animals

After burn induction, animals were divided into the following four groups (each group consisting of 5 rabbits). Group A: Treated with ointment of the plant extract (10% w/w). Group B: Treated with ointment of the plant extract using double dose regimen (20%w/w). Group C: Treated with a negative control containing ointment without the plant extract (placebo). Group D: Treated with Flamazine and considered as a positive control group.

The dressing were changed daily after cleansing the surface of burns with normal saline solution, and then the burn was wiped with the specified formula for each group along of 30 days. Formulae were applied with new gauze dressings held in position with a self Adhering wrap-around bandage (Diegelmann and Evans, 2004).

Rate of burn contraction

The burn contraction rate was measured as percentage reduction in wound size at every 10 days interval. Progressive decrease in the burn size was monitored periodically by tracing the boundary and the area was assessed graphically, the rate of burn contraction was determined according to the following formula (Brans et al., 1994).

$$C_B = \frac{A_0 - A_n}{A_0} \times 100\% \quad \text{.....Eq.2}$$

CB stands for the percentage of burn contraction, A0 is burnt area at zero time and An is burnt area at "n" day.

Immunohistochemistry study

Tissues were cut into small pieces (5x5x5mm) and immersed in 3.5% formalin solution until they were used. Tissues were dehydrated by several solutions of ascending concentrations of ethanol starting from 70% to 100% ethanol over two-hour period for each solution. Then other two cycles in xylene were applied over two-hour period using paraffin as dissolving solution. Subsequently, the tissues were infiltrated by using paraffin wax in an oven at temperature of 53-55oC for two hours. The tissues were embedded in paraffin wax at 53oC by the use of metal blocks. Then by the use of rotary microtome the tissues were sectioned at 5 μm. Some sections were stained with hematoxylin and eosin as described below. The other sections were stained with immunohistochemistry stains according to the procedure mentioned in section 2.8.

Staining by hematoxylin and eosin was fulfilled by placing the sections in two different xylene solutions, 5 min each. Then they were hydrated through different descending concentrations of ethanol solutions (100 % , 90 % , 80 %, 70%) for 3 min. After placing them in tab water, they were impregnated in Mayer' hematoxyline solution (5 min). subsequented with running tab water (5 min), eosin (3 min), ethanol solutions (70%, 80%, 90% , 95 % ,100% , 100%) 1 minute each , two cycles of xylene solutions (2 min each) , a drop of DPX was placed on the section , then covered with a cover slip and examined under light microscope.

Immunohistochemistry staining for skin tissue

Immunohistochemical detections of inducible nitric oxide (iNOS) and Heat Shock Protein (HSP70) were performed using commercially available mouse monoclonal antibody. The analysis was carried out according to the manufacturer instructions. Briefly, immunohistochemical detections of iNOS and HSP70 was demonstrated by using labelled streptavidin biotin LSAB kit, which consists of secondary biotinylated goat anti-mouse antibody and conjugated streptavidin horse radish peroxidase followed by 3',3'-Diaminobenzidine (DAB) chromogen. Sections (3-4 μm) were embedded on coated slides. Then they were deparaffinized in xylene twice for 2 min and rehydrated through serial dilution of alcohol (100%, 90%, 80%, and 70%) ended with water (2 min). Then the samples were treated under pressure with revealing solution for 2 min in Deckloking chamber (Biocare Company) in order to retrieve antigens and to block endogenous biotin. After cooling to room temperature, sections were incubated with 3% hydrogen peroxide in

methanol in order to block the endogenous peroxidase activity and then washed in phosphate buffer saline (PBS). According to the manufacturer's instructions, sections were incubated (1 hour at room temperature) with anti- iNOS and anti-HSP70.

After that they were washed in PBS and incubated with biotinylated secondary antibody for 15 min at room temperature and washed well with PBS. Sections were incubated with streptavidin horse raddish peroxidase for 15 min at room temperature and washed well with PBS. 3,3'-Diaminobenzidine chromogen were applied for 2 min or until the desired color intensity was developed, then washed with tap water to stop the reaction. Throughout the study, sections from normal skin known to express the investigated proteins were analyzed in parallel to serve as positive controls. Omission of the primary antibody from this sample was implicated as a negative control. All sections were counterstained with hematoxyline and examined by light microscope.

Slides were assessed using adopy photoshop software. Photos for sections were taken and divided into pixels. The total number of pixels was computed and represented both colours (blue and brown), then the brown colour (the colour of the marker under study) was computed and divided by the total number of pixels.

One way ANOVA was used to determine statistical significance between groups. P value < 0.05 was considered statistically significant difference.

Determination of antibacterial activity

Five different strains of bacteria including gram positive and gram negative were used in this study, namely: Escherichia coli (ATCC11229), Staphylococcus aureus (ATCC25923), Streptococcus pneumoniae (ATCC6303), Pseudomonas aeruginosa (ATCC2027) and Bacillus subtilis (ATCC6051). The organisms were obtained from the Department of Biological Science, Faculty of Science, University of Al al-Bayt, Jordan.

A dried powder of root of R. tinctorium (75 g) was extracted in 100 ml of distilled water for 48 h at room temperature with constant shaking using magnetic stirrer. The resulting mixture was then filtered through muslin cloth and the filtrate obtained was concentrated on a water bath. The resulting residue was reconstituted in distilled water in concentration of 150 mg/ml. Bacterial inoculation and incubation with extract nutrient agar and nutrient broth (oxoid) were prepared according to the manufacturers' recommendations. The agar-well diffusion method was used for the inoculation of bacteria. Plates containing 30 ml of sterile nutrient broth were inoculated with standardized inoculate (1.5 x 108 cells/ml) using sterile Pasteur pipette. Wells of 5 mm diameter were made at the centre of each plate and 0.15 ml of the various concentrations of the plant extract was dispensed into each well.

The extract was allowed to diffuse into the medium for 1hr at room temperature. Then it was incubated for 24 h at 37oC. The zones of growth inhibition was measured and recorded in millimeter. The negative control was set up in a similar manner except that the extract was replaced with sterile distilled water.

Determination of the minimum inhibitory concentration by the extracts of the plant at concentrations of 50, 100 and 150 mg/ml was carried out by the method described by National Committee for Clinical Laboratory Standard., 1990 (Marshall et al., 1996). About 0.1 ml of varying concentrations of the plant extract was introduced into the test tubes containing 0.9 ml of nutrient broth and standard bacterial cells. The test tubes were incubated at 37oC for 24 h. Controls were set up with the test organisms using distilled water instead of the plant extract. The minimum inhibitory concentration was taken as the tube with the least concentration of the extract has no visible growth after incubation.

The minimum bactericidal concentration of the plant extract was done according to the method highlighted in National Committee for Clinical Laboratory Standard., 1990 (Marshall et al., 1996); 1 ml from the mixture obtained in the determination of MIC stage was streaked out on the nutrient broth for 24 h. The least concentration of the extract with no visible growth was taken as the minimum bactericidal concentration.

Statistical analysis

Four groups each of them contains five rabbits were compared. Since the number of volunteers in each group is less than thirty and the number of groups exceeding two, ANOVA test was chosen as appropriate inferential tool of statistics. The test was carried out to compare a single parameter in each time therefore one way ANOVA was selected. P-value of 0.05 was used as a default value for such type of studies.

It was found that the rate of wound contraction is significantly different than the group of negative controls after 20 days of treatment. The expression of HSP70 and iNOS in treated groups are significantly different than the negative control group. Additionally, the antimicrobial property of the extract exhibits a positive effect with a statistical significance.

Results and discussion

Rate of burn contraction

The clinical evaluation showed visible differences in the process of burn healing after applying the above mentioned medication for each group of rats. As shown in Table 1, after 10 day, all treated animals groups exhibited increase in percentage of burn contraction as compared to control. On day 20, group B (double dose) animals showed a significant ($P< 0.05$) increase in the percentage of burn contraction followed by group A ,while group C

showed the lowest percentage of burn contraction as compared to the other treated groups. Day 30, the scars disappeared in group B and the contraction of burn was complete.

Topical application of R. tinctorum extract (20%w/w) at the contraction of burns resulted in a significant burn healing which may be due to its angiogenic and mitogenic potential. The enhanced rate of burn contraction might be due to enhancement epithelization facilitated by *R. tinctorum* ingredients.

Rabbits skin wound healing does not perfectly mimic human skin wound healing because the skin morphology is different (rabbits are described as loose-skinned animals). Loose skin allows wound contraction to play a significant role in closing the wound. Consequently, wound contraction, which is usually more rapid than epithelization, causes a reduction in the overall healing time of rabbit's wounds (Alvarez et al., 1983). On the other hand, humans have tight skin which makes the comparison with loose-skinned animals difficult. Although there are inherent drawbacks in using rabbits for comparisons with human skin wound healing, there are also advantages in the use of rabbits as a research model, such as the availability of a broad knowledge based on rabbits wound healing gained from years of previous research.

Immunohistochemistry studies

The results showed significant differences in expression of HSP70 between Group A and group C (P-Value = 0.002). When the dose was doubled in Group B, more expression of HSP70 was noted (P-Value = 0.001), while no significant differences were noted as a result of application of conventional treatment in Group D (P-Value = 0.378) (Figure 1).

The data showed that the expression of HSP70 was higher in group A and group B than group C and group D. On other hand the expression of iNOS was higher in group C and group D than group A and group B. These findings led the researcher to think that the positive outcomes for healing from burns is due to the over expression of HSP70. It has been reported that HSPs in general play significant roles as chaperones to maintain the structure of cellular proteins and to restore their normal function. HSPs have several beneficial roles in terms of enhancing growth and as anti-inflammatory (Desmettre et al., 2001; Beckham et al., 2004). Also the action of R. tinctorum is mediated through reducing the expression of iNOS which has been associated with cellular damage (Wilmink et al., 2007). Taken together, healing from burning has been improved due to the effect of treatment by R. tinctorum.

Antimicrobial activity

Table 2 showed diameter of zones of inhibition of bacterial growth at various concentrations of R. tinctorum. extract. The zone of inhibition increased significantly ($p <$

0.05) with increasing concentrations of the extract compared with positive control. The same pattern was observed for each of the other isolates. However, the various concentration of the plant extract produced wider zones of inhibition for S. aureus and B. substilis when compared to the zones of inhibition of gram-negative isolates.

(Table 2)

The anti-bacterial activity observed in this study was concentration-dependent. The various concentrations of the plant extract produced wider zones of inhibition for S. aureus and B. substilis when compared to the zones of inhibition of the gram-negative isolates. This implied that the gram-positive bacteria were more susceptible to the extract than the gram-negative bacteria, possibly because of the presence of outer membrane that serves as an effective barrier in gram negative species (Nikaido, 1999). The P. aeruginosa showed the least susceptibility to the extract. This could be due to the intrinsic resistance of P. aeruginosa due to the composition of the outer membrane barrier and Tran envelope multi-drug resistance pumps (Nikaido, 1999).

(Table 3)

MIC and MBC for the gram-positive bacteria were significantly lower ($p < 0.05$) than those of gram-negative bacteria. MIC values obtained for the entire test organisms were high, ranging from 25 to 100 mg/ml, when compared to the value of 10 mg/ml usually recorded for typical antibiotic. This difference may be due to the fact that the extract used was in the impure form.

Conclusion

It was concluded that *R. tinctorum* hexane extract has healing properties for burn injuries. The significant increase of HSP70 expression in treated groups inaddition to a reduction of iNOS expression are markers of healing promotion effect of the extract. Furthermore, *R. tinctorum* extract possesses antimicrobial properties against some pathogens attributed with wound infection such as *S. aureus* and *B. substilis.*

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Table.1 Comparisons of healed areas (cm²) (mean±SD) and healing percentages between tested groups and control groups. Group A: treated with extract *R. tinctorum (10%w/w).* Group B: treated with double dose of extract *R. tinctorum*. Group C: treated with placebo ointment. (negative control). Group D: treated with flamazine. On day 20, group B animals showed the highest percentage of burn contraction. On day 30, the scars disappeared in group B and the contraction of burn was almost completed.

Table.2 Inhibitory zones of bacteria growth (mm) on culture media by varying concentrations of aqueous extract of *R. tinctorum* values are means of four determinations ±S.E. *S.aureus and B.subtilis* are the most sensitive species to the extract among the tested bacteria.

Table.3 Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) for the extracts of *R. tinctorum* (mg/ml). *S.aureus and B.subtilis* are the most sensitive species to the extract among the tested bacteria

Figure.1. The expression of HSP70 and iNOS among study groups. Group A: treated with extract *R. tinctorum*. Group B: treated with double dose of extract *R. tinctorum*, Group C: treated with placebo ointment (negative control). Group D: treated with flamazine (positive control). The extract exhibits strong induction of HSP70 expression and significant inhibition of iNOS enzyme expression in comparison with flamazine as a positive control.

Table 1

Groups	10 days	20 days	30 days
A	7.4±1.3 (12%)	27.6 ± 0.9 (46%)	50.4±0 .7 (84%)
B	6.9 ± 0.7 (11%)	33.20.6* (55%)	56.6± 1.1* (93%)
C	4.8±1.1 (8%)	24±1.1 (40%)	45± 0.4 (75%)
D	6±0.8 (10%)	25.8± 1.3 (43%)	49.2± 1.4 (82%)

(*)Significant difference ($p<0.05$) in comparison with the other groups in the same column.
Healing was considered complete where percentage of healing area >90%.

Table 2

Concentration (mg/ml)	*S.aureus*	*B.subtilis*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*
Ext.50	7.00 ±0.29	6.00 ±0.29	5.20 ±0.12	5.10 ±0.12	5.10 ±0.15
Ext.100	10.80 ±0.12	10.00 ±0.29	7.00 ±0.17	5.10 ±0.15	5.00 ±0.12
Ext.150	14.83 ±0.20	12.10 ±0.15	10.10 ±0.20	9.00 ±0.17	6.10 ±0.15
Flam. 10	20.45±0.12	16.65±0.13	21.77±0.12	14.98±0.20	18.87±0.20

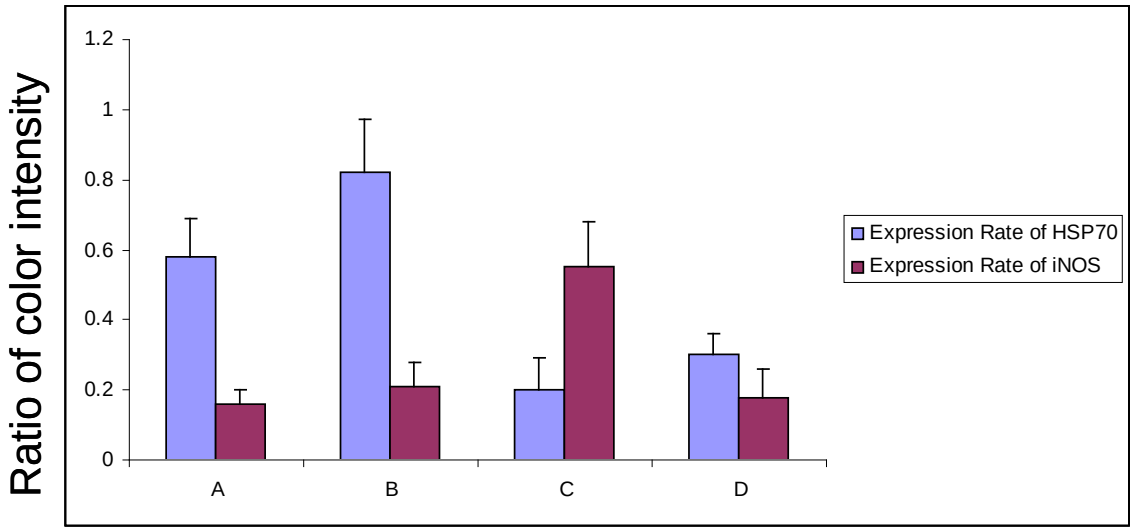
Ext. and Flam. are abbreviations of extract and flamazine respectively.
Sensitive species depicted at the mid concentration (100mg/ml) at least half inhibitory zone of Flamazine (10mg/ml).

Table 3

	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*
MIC	25.00 ±5.00	48.00 ±5.00	100.00 ±5.00	100.0 ±5.00	100.50 ±5.00
MBC	90.00 ±5.00	100.00 ±5.00	130.00 ±5.00	150.00 ±5.00	150.00 ±5.00

MIC is minimum inhibitory concentration. MBC is minimum bactericidal concentration.
Species with MIC ≤ 50 mg/ml and MBC ≤ 100 mg/ml were considered sensitive.

Figure 1



Preparations which increased ratio of HSP70 pigmentation at the specimen more than 20% were considered healing enhancers.
Preparations which decreased ratio of iNOS pigmentation at the specimen to less than 55% were considered healing enhancers.