

## Research Article

# MODULATION OF THE INFLAMMATORY CYTOKINES AND CYTOPROTECTIVE ENZYME BY BILIRUBIN TREATMENT TO ENHANCE CUTANEOUS WOUND HEALING IN RATS

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**ABSTRACT:** Inflammation is the main process of wound healing where expression of certain cytokines likes Interleukin-10 (IL-10) and Tumour necrosis factor  $\alpha$  (TNF  $\alpha$ ) plays an important role. In view of the antioxidant potential of bilirubin, the present study was aimed to evaluate time-dependent (day 3, 7, 14) wound healing effects of bilirubin ointment (0.3%) in excisional wound model in rats. Thirty-six acclimatized healthy male Wistar rats (120-150g) were divided into control and treated groups containing 18 rats each. Each group was further sub- divided into three sub-groups (day 3, 7 and 14 days, n= 6). The ointment base (soft paraffin 90%, lanolin 5% and hard paraffin 5%) and bilirubin ointment (0.3%) were applied topically once daily for 14 days in control and treated group respectively. The wound area was determined on days 3, 7, and 14. The mRNA expression of TNF  $\alpha$  gene and IL-10 gene were determined on days 3, 7 and 14 by Real Time PCR and their protein levels by ELISA method. The protein expression of cyto-protective enzyme HO-1 (Heme oxygenase-1) and growth factor VEGF (Vascular growth factor) was determined by western blotting method. The mRNA expression and protein level of TNF  $\alpha$  was significantly reduced and IL-10 was significantly increased whereas the expression of HO-1 enzyme and VEGF was significantly increased in treated group on days 3, 7 and 14. It may be concluded that the bilirubin has pro-healing potential.

**Key words:** Bilirubin, Ointment, Cytokines, Wound healing, Rat.

## INTRODUCTION

Wound healing is a complex physiological process which occurs after injury and can be best described as a complex programmed sequence of cellular and molecular processes which include haemostasis, inflammation, cell migration, angiogenesis, provisional matrix synthesis, deposition of collagen and re-epithelialization (Lazarus *et al.* 1994, Pierce and Mustoe 1995). During hemostasis, neutrophils and monocytes/macrophages enter the wound site. The influx of these cells at wound site is beneficial, since they play an important role in the defence against contaminating bacteria by phagocytosis and by the production and release of various proteinases and reactive oxygen species (ROS). The activated neutrophils and tissue macrophages use an NADPH cytochrome b-dependent oxidase for the reduction of molecular oxygen to superoxide anions (Wlentjes and Segal 1995).

In addition to these inflammatory cells, other cell types, such as, fibroblasts can also be stimulated to produce reactive oxygen species in response to pro-inflammatory cytokines (Meier *et al.* 1989). In later stage of wound healing, keratinocytes and fibroblasts are stimulated to proliferate and migrate over the provisional matrix to form the granulation tissue. During the final phase of wound healing, the granulation tissue is replaced with an acellular scar, when myo-fibroblastic and vascular cells in the wound undergo apoptosis (Falanga 2005).

The cyto-protective enzyme HO-1, strongly induced by pro-oxidants and inflammatory agents, degrades heme to three compounds: CO, free iron (Fe<sup>2+</sup>) and biliverdin which is subsequently reduced to bilirubin by NADPH dependent biliverdin reductase (Wagner *et al.* 2003). Bilirubin at a low concentration scavenges reactive oxygen species in vitro, thereby, reducing oxidant-

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mediated cellular damage and attenuating oxidative stress *in vivo* (Stocker *et al.* 1987). The roles of biliverdin and bilirubin in counteracting oxidative and other stresses have been reviewed extensively (Foresti *et al.* 2004, Morse and Choi 2005). Bilirubin induces HO-1 synthesis and acts as anti-inflammatory agent by reducing expression of pro-inflammatory cytokine TNF  $\alpha$  and increasing the expression of anti-inflammatory cytokine IL-10 (Hongjun *et al.* 2006, Ahanger *et al.* 2016). HO-1 increases angiogenesis by increasing vascular endothelial growth factor (VEGF) synthesis (Jozkowicz *et al.* 2002). Neovascularization plays a central role in wound granulation and is required for normal healing (Sen *et al.* 2002, Martin *et al.* 2003).

Despite some recent advances in understanding the basic principles, problems in wound healing continue to cause significant morbidity and mortality (Fine and Mustoe 2006). In view of above facts, the present study was designed to investigate the time-dependent effect of bilirubin in excisional wound model in rats on topical application.

## **MATERIALS AND METHODS**

### **Animals**

Thirty six healthy male Wistar rats (120-150g) (six-week-old) were procured from Laboratory Animal Resource Section of IVRI, Izatnagar, Bareilly). All the animals were acclimatized for a period of 7 days prior to the commencement of the experiments. The rats were divided into two groups, control and treated containing 18 rats each. Each group further sub-divided into three sub-groups (day 3, 7 and 14, n= 6). The ointment base and bilirubin ointment (0.3% in ointment base) were applied topically once daily for 14 days in control and treated group respectively. The wound area, m-RNA expression and protein level of cytokines TNF  $\alpha$  and IL-10, Western blotting of HO-1 and VEGF were estimated on days 3, 7 and 14 post-wounding. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee [(No.F.26-1/2010-11/JD (R)] of IVRI, Izatnagar, Bareilly, U.P. and conforms to the Guide for the care and use of laboratory animals (1996).

### **Wound model**

The animals were anesthetized by an intraperitoneal (i.p.) injection of pentobarbitone sodium (50 mg/kg). Their dorsal surface was shaved and wiped with 70% ethanol. A 2x2 cm<sup>2</sup> (~ 400 mm<sup>2</sup>) full thickness open excision-type wound was created. Animals, after recovery from anaesthesia, were housed individually in properly disinfected cages.

### **Preparation of ointment**

The bilirubin (Sisco Research Laboratories Pvt. Ltd, Mumbai) (0.3%) ointment was prepared in simple ointment base containing soft paraffin (90%), hard paraffin (5%) and lanolin (5%) on ointment slab.

### **Measurement of wound area**

The wound area was measured planimetrically on pre-determined time intervals on days 3, 7 and 14 post-wounding. During the measurement of wound area, animals were gently but firmly held on a table top with the wound facing upwards. A firm but flexible transparent polythene rectangular (3 x 3 cm<sup>2</sup>) sheet was held just over the wound and its margins were marked with a permanent marker on sheet and the animal was released back to the cage. The area demarcated on the transparent sheet was estimated by planimetrically in which a standard quality card paper was used to convert the area of the wound on the transparent sheet into the weight of the card paper with same area. The weight of the card paper/unit area was already known, therefore, the weight of each card paper for a particular wound was estimated easily.

### **Tissue harvesting**

The animals were sacrificed on days 3, 7 and 14 with an overdose of diethyl ether (Ahanger *et al.* 2011) to collect granulation tissue which was immediately divided into two parts. One part was kept in RNA later™ (Qiagen, USA) and stored at -20°C until RNA extraction for quantitative real time-PCR. The 2<sup>nd</sup> portion was snap frozen in liquid nitrogen and the tissue homogenate of the same was prepared with the help of motor homogenizer at 4°C in ice-cold lysis buffer [100 mg tissue in 1ml lysis buffer: 1% Triton X 100, 10 mM phenyl methyl sulfonyl fluoride (PMSF), 1mg/ml aprotinin and 1 mg/ml leupeptin in phosphate buffer saline (PBS) pH 7.4]. The homogenates were then incubated at 4°C for 30 min and centrifuged at 12,000 rpm for 10 min at 4°C. The aliquots of the supernatant were prepared and stored at - 80°C till further processing for Western blotting and enzyme-linked immunosorbent assay (ELISA).

### **Isolation of RNA and real-time polymerase chain reaction for m-RNA expression**

The granulation tissue excised on days 3, 7 and 14 post-wounding was used to study the expression pattern of mRNA of growth factors (TNF  $\alpha$ , IL-10). Total RNA was extracted using the method described by reagents supplier (Ribozol™ RNA extraction reagents, Amresco LLC, USA) using thawed tissue from RNA later® and quantified by nano-spectrophotometer. cDNA synthesis

**Table 1. Description of primers used.**

Gene	Primer Sequences	Produ-ct size	Annealing Temp.	Accession Number
<b>GAPDH</b>	F: 5'-AACTTTGGCATTGTGGAAGG-3' R: 5'-ACACATTGGGGGTAGGAACA-3'	452	60 °C	NM028301.1
<b>TNF <math>\infty</math></b>	F: 5'- GGCCACCACGCTCTTTCTGTCA-3' R 5'-TGGGCTACGGGCTTGTCACCTC-3'	153	60 °C	NM012675.3
<b>IL-10</b>	F: 5'-CCTGCTCTTACTGGCTGGAG -3' R: 5'-TGTCCAGCTGGTCCTTCTTT-3'	161	60 °C	NM012854.2

was carried out from the total RNA using cDNA synthesis kit (FERMENTAS, USA). An aliquot (1  $\mu$ l) of cDNA was used as a template for the subsequent real time PCR. The real time PCR assay was performed by using 2 X QuantiTect SYBR Green PCR Master Mix, (Qiagen, USA) in 96 well plate of Stratagene Q-Cycler and analyzed by using Mx 3000 P software. The real-time PCR experiment was carried out according to the manufacturer's instructions and the following thermal cycling profile was used (40 cycles): 95°C for 15 min, 94°C for 15 s, (temperature varies depending on primer) for 30 s and 72°C for 30 s. The primers used are given in Table 1. The  $\Delta\Delta$ CT method of relative quantification was used to determine the fold change in expression and this was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same samples ( $\Delta$ CT=CT<sub>Target</sub>-CT<sub>GAPDH</sub>). Further, it was normalized with the control ( $\Delta\Delta$ CT= $\Delta$ CT<sub>Treatment</sub> -  $\Delta$ CT<sub>Control</sub>). The fold change in expression was then obtained as  $2^{-\Delta\Delta$ CT (Livak and Schmittgen 2001).

#### Western blot analysis

The equal volume of 2X lamilliae sample buffer was mixed in frozen tissue extracts already thawed on ice. Then they were heated at 100°C for 10 min and allowed to cool at 4°C for 20 min. The samples were then loaded on 12–15% tris-glycine, sodium dodecyl sulfate-polyacrylamide gels for fractionation at 150 V. Predetermined and pre-stained molecular weight standard (Fermentas) was used as marker. After subjecting protein under reducing conditions on polyacrylamide gel, they were transferred to polyvinylidene difluoride (PVDF) membrane at 150 mA for 4 h. The non-specific sites on the membrane were blocked with blocking buffer (5% skimmed milk powder in 0.5 % PBS-T) at 37°C for 1 h. Then the membranes were washed thrice with washing buffer by repeated agitation for 5 min. Immunodetection

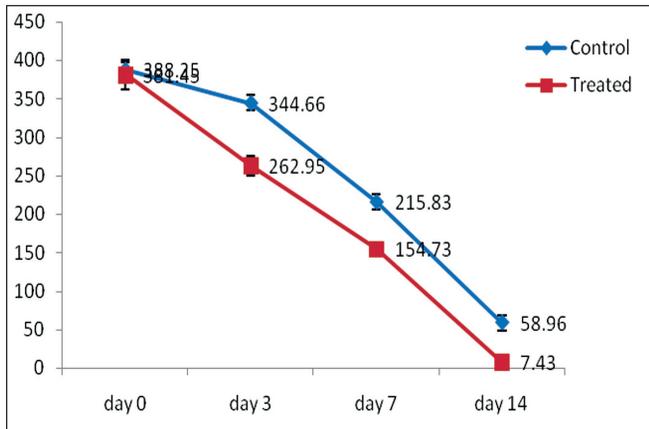
of the GAPDH, HO and VEGF were carried out using respective goat polyclonal antibodies, GAPDH goat polyclonal IgG antibody (cat. sc-20356, 1:500 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), HO-1 goat polyclonal IgG antibody (cat. sc-1796, 1:500 Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and VEGF goat polyclonal IgG-EG-VEGF antibody (cat. sc-30343; 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C overnight. After washing with PBS-T (phosphate buffer saline Tween-20), the membranes were incubated with secondary antibody; HRP-conjugated chicken anti-goat IgG (cat. sc-2953; 1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1-2 h at 37°C. Following successive washes, the colour reaction were carried out using the diaminobenzidine (DAB) system. The blots were subsequently scanned and band intensity was quantified by densitometry software (Image J, NIH). The western blot data for HO-1 and VEGF were corrected for corresponding GAPDH values and the results were expressed as normalized protein levels. A minimum of four such blots were performed for every protein analysed.

#### ELISA

The protein levels of TNF  $\infty$ , IL-10 estimated by ELISA assay as per the ELISA kit manufacturer's instructions in granulation tissue lysates. The assay of IL-10 (Genetix Biotech Pvt, Ltd) and TNF  $\infty$  (Komabiotech Pvt Ltd) was done using a 96-well microtiter plate reader (photometry at 450 nm). Each sample was used in duplicate and OD measurements were then verified against a standardized curve. The results were expressed as pg/ml/100 mg of tissue.

#### Statistical Analysis

Results are expressed as mean  $\pm$  S.E.M. Data were analyzed by Bonferroni's post test and unpaired student 't' test using Graph Pad Prism Version v4.0 (Graph Pad Software, San Diego, CA). Values on the same days of



**Fig. 1. Effect of topical application of bilirubin ointment (0.3%) on wound area on days 3, 7 and 14 in rats (n= 6).**

control and bilirubin-treated groups were compared and probability level of less than  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

### Effect of bilirubin (0.3%) on wound contraction on day 0, 3, 7 and 14 post- wounding

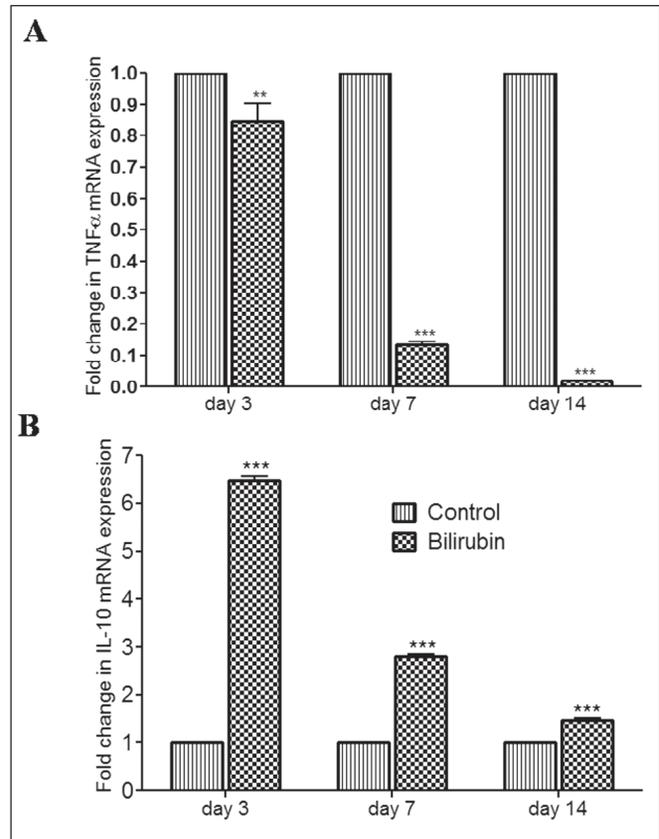
Fig. 1 shows actual wound area ( $\sim$  mm<sup>2</sup>) on day 0, 3, 7 and 14 post-wounding of control and bilirubin treated rats. As evident from table the significant difference in area was observed in bilirubin vs control treated on day 3 ( $262.95 \pm 14.12$  mm<sup>2</sup> and  $344.66 \pm 9.75$  mm<sup>2</sup>), day 7 ( $154.73 \pm 19.19$  mm<sup>2</sup> and  $215.83 \pm 10.55$  mm<sup>2</sup>) and day 14 ( $7.43 \pm 2.69$  mm<sup>2</sup> and  $58.96 \pm 4.06$  mm<sup>2</sup>). The significant reduction in wound area was observed in treated rats as compared to control rats.

### Effects of bilirubin treatment on mRNA expression of genes TNF $\alpha$ and IL-10

The mRNA expression (fold changes over control) was depicted in bilirubin-treatment significantly ( $P < 0.001$ ) decreased of the TNF  $\alpha$  and IL-10 (Fig. 2A-B). The bilirubin treatment significantly decreased mRNA expression of the TNF  $\alpha$  on day 3 ( $0.85 \pm 0.06$ ) continued up to day 7 ( $0.14 \pm 0.01$ ) and day 14 ( $0.02 \pm 0.00$ ). The IL-10 mRNA expression was significantly higher than the control group on day 3 ( $6.49 \pm 0.090$  fold changes over control), day 7 ( $2.80 \pm 0.04$  fold changes over control) and day 14 ( $1.47 \pm 0.05$  fold changes over control).

### Effects of bilirubin treatment on protein levels of TNF $\alpha$ and IL-10

The protein levels of TNF  $\alpha$  and IL-10 are presented in Fig. 3 A-B. The TNF  $\alpha$  protein level significantly decreased on day 3 post-treatment ( $520.83 \pm 15.10$  pg/ml/100mg of tissue), as compared to control ( $642.45 \pm$



**Fig. 2. Effects of topical application of bilirubin on relative mRNA expression of TNF  $\alpha$  (A) and IL-10 (B) in healing tissue of excision wounds in rats.**

The mRNA expressions of these were normalized by GAPDH at each time point and data are expressed as means  $\pm$  S.E.M. \*\* $P < 0.01$ , \*\*\*  $P < 0.001$  versus ointment base-treated group on same day (n=6).

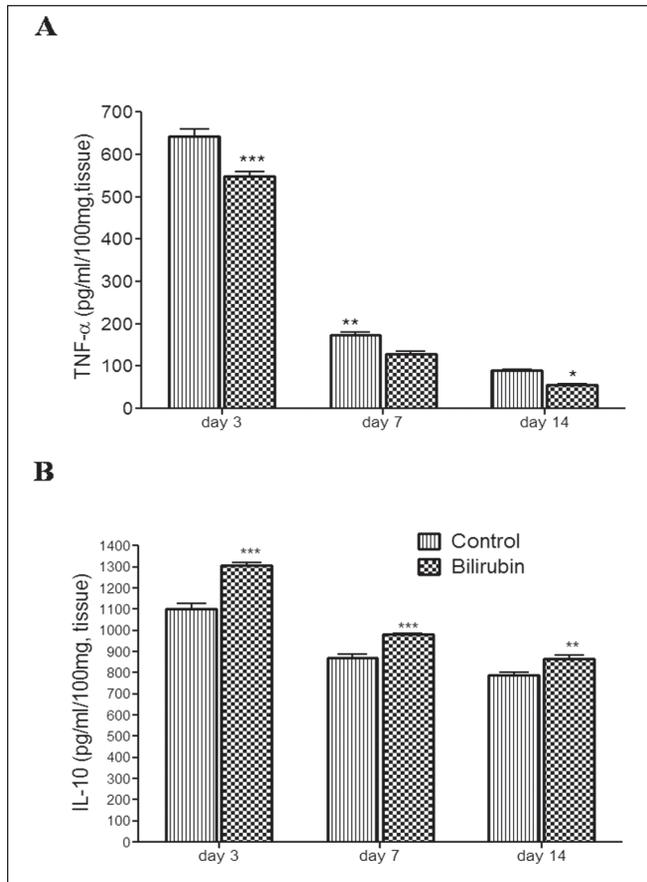
17.41 pg/ml/100mg of tissue) and subsequently reduced on days 7 and 14.

The protein level of IL-10 revealed significantly higher ( $1308.14 \pm 13.74$  pg/ml/100mg of tissue) on day 3 in bilirubin treated as compared to control animals ( $1100.66 \pm 25.30$  pg/ml/100mg of tissue) (Fig. 4B). This trend was maintained on day 7 and 14 in treated rats.

### Effects of bilirubin treatment on protein expression of HO-1 and VEGF

Fig. 4 A-C depicts the representative blots for GAPDH, HO-1 and VEGF of days 3, 7 and 14. Bilirubin significantly ( $P < 0.001$ ) increased the fold expression of HO-1 protein ( $3.54 \pm 0.08$  and  $2.427 \pm 0.165$ ) and VEGF protein ( $5.45 \pm 0.27$  and  $4.070 \pm 0.321$ ), respectively, on days 3 and 7 compared to control. On day 14, HO-1 and VEGF expression were comparatively low, as compared to initial period of wound healing.

In the present study, topical application of bilirubin (0.3%) ointment significantly increased per cent wound closure in time-dependent manner by modulating pro and



**Fig. 3. Effects of topical application of bilirubin (0.3%) on protein levels of TNF  $\alpha$  (A) and IL-10 (B) in granulation tissue on days 3, 7 and 14 post-wounding.**

Data are expressed as mean  $\pm$  SEM, (\* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001) (n=6).

anti-inflammatory cytokines (TNF  $\alpha$ , IL-10) as well as growth factor (VEGF) and cyto-protective enzyme (HO-1). The mRNA expression of TNF  $\alpha$  markedly reduced whereas expression of IL-10, VEGF and cyto-protective enzyme HO-1 significantly increased on topical application of bilirubin ointment on days 3 and 7. These results are strongly supported by protein analysis.

Upon injuries, the HO-1 pathway is activated, thereby, there is release of CO, free iron (Fe<sup>2+</sup>) and biliverdin which is subsequently reduced to bilirubin (Wagner *et al.* 2003). Bilirubin potentially attenuates reactive oxygen species and reactive nitrogen species which are invariably present in the microenvironment of wounds and have anti-inflammatory property and therefore, presence of bilirubin at low concentration has been suggested to synergize the healing process (Stocker *et al.* 1987, Foresti *et al.* 2004, Morse and Choi 2005). The biliverdin and bilirubin preserved endothelial cell integrity (Sedlak and Snyder 2004) and prevented endothelial cell death and sloughing (Rezzani *et al.* 2003, Mc Clung *et al.* 2004).

TNF  $\alpha$ , a macrophage derived pro-inflammatory

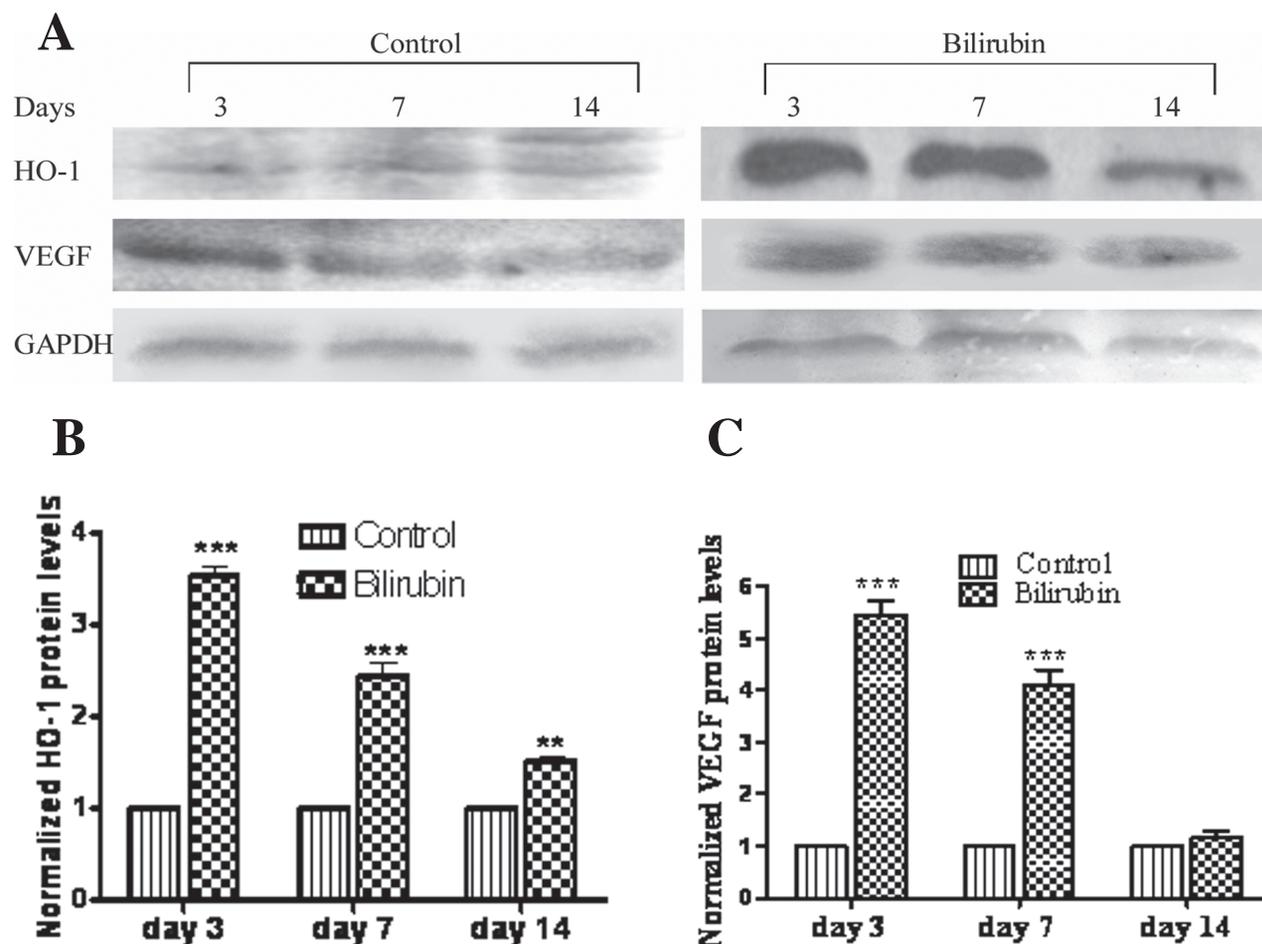
cytokine is known to play a major role in the inflammatory phase of wound healing (Rosenberg and Torre 2006). In this study, at the end of inflammatory phase the bilirubin-treated group has significantly reduced levels of TNF  $\alpha$ . It is, thus, apparent that bilirubin during the first stage of healing, decreased the TNF  $\alpha$  production (Ahanger *et al.* 2016).

In the present study, bilirubin significantly elevated IL-10 protein level and increased mRNA expression on days 3, 7 and 14. IL-10 influenced the wound-healing environment by decreasing the expression of pro-inflammatory/pro-fibrotic mediators, resulting in decreased recruitment of inflammatory cells to the wound (Ribbons *et al.* 1997) and also suppressed TNF  $\alpha$  production. IL-10 mRNA expression and levels of protein in wound tissue were significantly higher in bilirubin-treated rat. Thus, the findings suggest that bilirubin regulated anti-inflammatory and pro-inflammatory cytokines.

The main angiogenic and vasculogenic mediator necessary for rapid and effective tissue repair is vascular endothelial growth factor (VEGF). In this study, the mRNA expression of VEGF was significantly higher on days 3 and 7 in bilirubin-treated wounds and subsequently low on day 14. The VEGF protein expression was highest on day 3. These VEGF expression peaks coincide with the clinical appearance of granulation tissue (Asahara *et al.* 1999). It is demonstrated that biliverdin up-regulates VEGF production in endothelial cells (Jozkowicz *et al.* 2002) and keratinocytes (Jazwa *et al.* 2006). VEGF also improved angiogenesis during wound healing by stimulating the migration of endothelial cells through the extracellular matrix (Ferrara 1999).

HO-1 is an inducible enzyme and its expression is rapidly increased after tissue injury, and it has definite role in wound healing as pro-angiogenic, anti-apoptotic, cytoprotective and anti-inflammatory agent. The pro-healing role of HO-1 has also been confirmed by inhibition of HO-1 through the use of tin protoporphyrin-IX which led to retardation in the closure of wound in rats and mice (Przeczek-Grochot *et al.* 2009). In our study, the higher mRNA and protein expression of HO-1 in bilirubin-treated wounds corroborate earlier reports (Hanselmann *et al.* 2001, Kamfer *et al.* 2001). This is also supported by observation that bilirubin/biliverdin has salutary effects on islet survival based on the suppression of inflammation by up-regulation of protective gene HO-1 (Hongjun *et al.* 2006). The early and fast wound healing was observed in excision wound model of rats by modulating HO-1 expression (Ahanger *et al.* 2010, Ahanger *et al.* 2011).

It can be concluded that the treatment of cutaneous excision wounds with bilirubin ointment showed a potent



**Fig. 4. Representative Western blots of HO-1, VEGF and GAPDH.**

(A) from the samples of control and bilirubin- treated animals on days 3, 7 and 14. Effect of topical application of ointment base on relative expressions of HO-1 (B) and VEGF (C). The protein expression as normalized by GAPDH at each time point and data are expressed as means  $\pm$  S.E.M. (\*\* P<0.01 and \*\*\* p<0.001) versus control on the same day (n=4).

pro-healing activity which may be attributed to its pro-inflammatory in early stage (TNF  $\infty$ ) and anti-inflammatory effects by modulating cytokines (IL-10). The synergistic effects were shown by cyto-protective effect by synthesizing HO-1 enzyme, stimulation of growth factor (VEGF) indicated by complete re-epithelialization, better angiogenesis and deposition of collagen fibres, thereby, favouring a fast and early healing process in treatment group.

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## Research Article

# CYTOTOXICITY STUDY OF *FICUS RELIGIOSA* LEAF EXTRACT AGAINST JURKAT CELL LINE

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**ABSTRACT:** Different parts of *Ficus religiosa* tree have traditional usage against many ailments and diseases. In the present study, 85% ethanol extract of *F. religiosa* leaves were partially purified by PLC Silica gel 60 F<sub>254</sub>, 2 mm, 20X20 cm preparative thin layer chromatography using toluene and ethyl acetate (8:2, v/v) as solvent. Fraction 7 (FR7) showed highest brine shrimp larvicidal activity as regard to the leaf extract of *F. religiosa*. *In vitro* anticancer potential of same fraction against Jurkat cell line was investigated by means of flow cytometry using annexin V/7AAD apoptosis kit. *F. religiosa* leaf extract was found to markedly inhibit the proliferation of Jurkat cells in a dose-dependent manner.

**Key words:** *Ficus religiosa*, Cytotoxicity, Apoptosis, Jurkat cell line.

## INTRODUCTION

Plant-derived polyphenolic compounds such as flavonoids, tannins, curcumin and the stilbene resveratrol are recognized as naturally occurring antioxidants and have been implicated as anticancer compounds (Mukhtar *et al.* 1988). In recent years, several reports have documented that plant polyphenolics, including curcumin, resveratrol and gallo-catechins such as gallic acid, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG), induce apoptosis in various cancer cell lines. Most of the current anticancer drugs are derived from plant sources, which act through different pathways converging ultimately into activation of apoptosis in cancer cells leading to cell cytotoxicity. Several plant products have been tested for antitumor activity and some of these, such as vincristine (from Madagascar periwinkle, *Catharanthus roseus*) and taxol (from the Pacific yew tree, *Taxus brevifolia*) are now available as drugs of choice (Kwiecinski *et al.* 2008).

*Ficus religiosa* or peepal tree or Sacred Fig or *Asvatha*

is a large deciduous tree with few or no aerial roots. The tree is distributed throughout India. Recently Hyo *et al.* (2008) reported antioxidant property of methanolic leaf extract of *F. religiosa*. Patil *et al.* (2010) reported promising immunostimulant properties of ethanolic extract of leaves of *Ficus carica* (Fig tree) in mice. There are meager studies on anti-cancer activity of the tree parts. Lately, Uddin *et al.* (2011) has reported cytotoxic activity of *F. religiosa* leaves.

The present study aimed at investigating the cytotoxicity property of aqueous leaf extract of *Ficus religiosa* against Jurkat cell line (human T-cell leukemia) *in vitro*.

## MATERIALS AND METHODS

Plant samples (*Ficus religiosa* L, Family: Moraceae) were collected in and around Bhubaneswar during winter season (November-December). The plants were authenticated by the botanists, OUAT, Bhubaneswar. After collection, leaves were cleaned, shade-dried and

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then powdered. A voucher specimen (Accession number 20104) representing this collection has been deposited in the department for further reference.

### Preparation of plant sample

Five grams of the powder was extracted with 100 ml solvent by keeping on a rotary shaker overnight. An 85% ethanol solution was used as solvent of extraction. Extracts were filtered through Whatman No. 1 filter paper and filtrates were dried using a rotary evaporator at 40°C.

### Purification of extract by HPTLC

HPTLC - UV analysis was performed on a computerized densitometer scanner 3, controlled by *winCATS* planar chromatography manager *version 1.4.2*. (CAMAG, Switzerland), having the facility of multiwavelength Scanning. The precoated TLC plate of silica gel 60 F<sub>254</sub> of dimension 20 x10 cm and 0.2 mm thickness (EMerck, Germany) was used for fingerprinting study. Toluene and ethyl acetate were used as mobile phase (developer) in the ratio of 8:2. Chromatography procedure described by Kowalska *et al.* (2002) was followed.

For isolation of different fractions from the extracts, preparative thin layer plates (PLC Silica gel 60 F<sub>254</sub>, 2 mm, 20 x 20 cm, EMerck, Germany) were used. Samples were applied across the entire width of the plate and different bands were scraped from the plate after UV detection. For elution of compounds, 85% ethanol was mixed with the scrapings separately and filtered with Whatman filter paper. After evaporation of ethanol, the fractions were lyophilized and the residue was weighed.

### Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was used to screen the cytotoxic activity of the fractions collected after preparative thin-layer chromatography (Meyer *et al.* 1982). For the experiment, lyophilized fractions were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 µg/ml using simulated seawater. The solutions were then added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 ml simulated seawater. After 24 h, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 s of observation. Vincristine sulphate was used as positive control.

### Apoptosis study

The assay methodology followed is based on utilizing annexin V to detect the cell surface exposure of phosphatidylserine (PS) that occurs during apoptosis

(Koopman *et al.* 1994, Vermes *et al.* 1995).

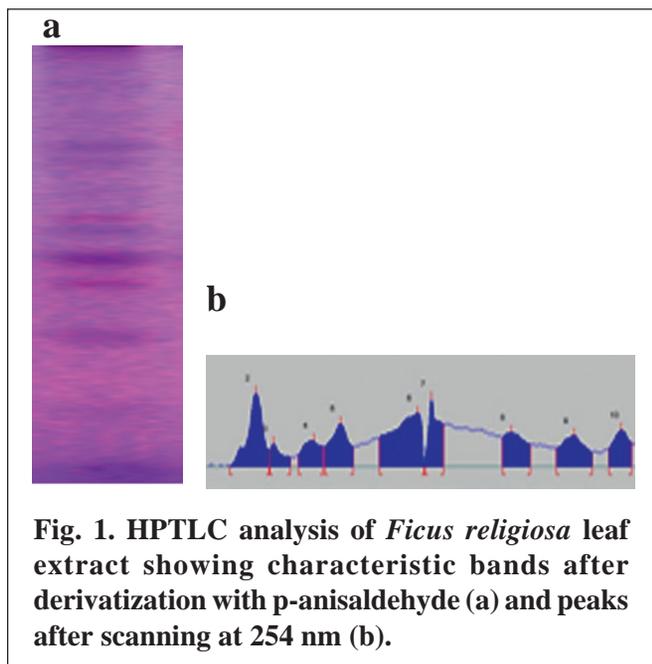
Jurkat cells were collected from ATCC (American Type Culture Collection). Cells were cultured in IMDM (Iscove's Modified Dulbecco's Media) with 10% FBS in presence of antibiotics, L-Glutamine and were maintained in a CO<sub>2</sub> incubator at 37°C with 5% CO<sub>2</sub>. Cells were plated in a 24-well culture (Nunc) plates at 0.5 X 10<sup>6</sup> cells per ml. Fractions having the highest brine shrimp larvicidal activity were dissolved in water to obtain a concentration of 100 µ/200 µ. The aqueous samples were diluted in media and were added at indicated different doses (0.01, 0.1, 1, 10 and 100 µ/ml). The cells were incubated for 24 hours at 37°C in presence of 5% CO<sub>2</sub>. Equivalent amount of media alone was added to the control wells. The efficacy of the samples to induce cell death was determined by annexin V/7AAD apoptosis kit (BD Biosciences). The samples were subjected to flow analysis, data were acquired using FACS Calibur (BD Biosciences) and were analyzed using CellQuestPro software (BD Biosciences).

## RESULTS AND DISCUSSION

HPTLC, like HPLC and GC, is suitable for development of chromatographic fingerprint to determine and identify complex herbal extracts (Chen *et al.* 2006). Extracts of *F. religiosa* leaves has given eight (Rf value 0.14, 0.17, 0.22, 0.26, 0.38, 0.47, 0.56 and 0.72) visible bands after derivatization with p-anisaldehyde. Same numbers of peaks were also obtained after scanning with UV light at 254 nm (Fig. 1). Recent study by Rathee *et al.* (2015) has validated TLC densitometric quantification of the two marker compounds (stigmaterol and lupeol having Rf value 0.37 and 0.60 respectively) from methanolic extract of *F. religiosa* fruits using the solvent system of toluene: methanol (9:1, v/v). But, this could not be compared with the present study where

**Table 1. Brine shrimp lethality bioassay of different purified fractions of *F. religiosa* leaf extracts (10 µg/ml concentration) with vincristine sulphate as positive control.**

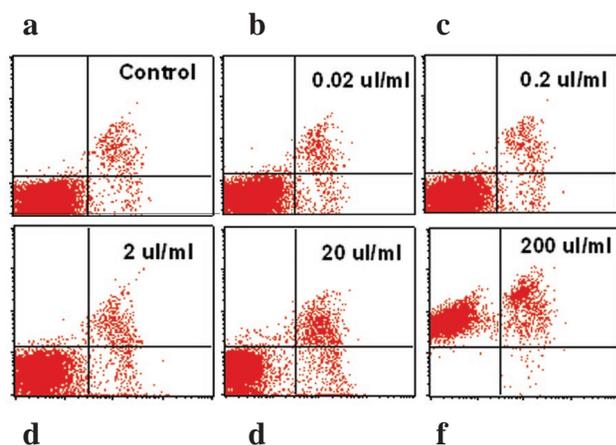
F. religiosa leaf extract		Vincristine sulphate	
Fractions	% mortality	Concentration	% mortality
FR1	43.19 ± 1.18	40 µg/ml	100
FR2	30.52 ± 2.27	20 µg/ml	100
FR3	37.95 ± 1.64	10 µg/ml	100
FR4	42.03 ± 2.08	5 µg/ml	93 ± 4.03
FR5	36.97 ± 1.88	2.5 µg/ml	75 ± 3.77
FR6	29.81 ± 2.02	1.25 µg/ml	62 ± 3.71
FR7	100	0.63 µg/ml	49 ± 2.08
FR8	35.47 ± 2.47	0.31 µg/ml	41 ± 2.83



toluene: ethyl acetate (8:2) was used as mobile phase for HPTLC fingerprinting of 85% ethanolic extract of *F. religiosa* leaves.

Out of all the fractions tested for cytotoxicity, fraction 7 (FR7, Rf value 0.56) showed highest brine shrimp larvicidal activity as regard to the leaf extract of *F. religiosa* (Table 1). Therefore, this particular fraction having the highest cytotoxic activity was selected in the next step for further investigation.

The results of flow cytometry assay using Jurkat cell line showed that FR7 of *F. religiosa* extract has apoptotic activity against cancer cells. At lower doses, it did not induce any cell death, but when used at higher doses, Jurkat cells had undergone apoptosis, as it showed that a significant number of cells have become positive both



(a. control; b. 0.01  $\mu\text{g/ml}$ ; c. 0.1  $\mu\text{g/ml}$ ; d. 1  $\mu\text{g/ml}$ ; e. 10  $\mu\text{g/ml}$  and f. 100  $\mu\text{g/ml}$ ).

for annexin V and 7AAD. At a dose of 200  $\mu\text{l/ml}$  (*i.e.* 100  $\mu\text{g/ml}$ ), all cells died and this suggests that at higher dose, fraction 7 of *F. religiosa* extract was able to induce apoptosis in Jurkat cells (Fig. 2).

Leaves of *F. religiosa* contain lupeol which is reported to have anticancer activity (Chaturvedi *et al.* 2008; Nigam *et al.* 2009). Uddin *et al.* (2011) reported cytotoxic activity (50% inhibition of cell growth,  $\text{mg/ml}$ ) of *F. religiosa* leaves against normal mouse fibroblasts (NIH3T3), gastric cancer (AGS), colon cancer (HT29) and breast cancer (MDA-MB-435S) cells to be 1.01, 2.16, >2.50, >2.50 for methanolic extracts; whereas >2.50, >2.50 for aqueous extracts respectively. The present study supported the above observation. However, the cytotoxicity of *F. religiosa* leaf extract is less, as compared to that of Vincristine.

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