

PHARMACOGNOSTIC EVALUATION OF TURMERIC (*CURCUMA LONGA*) EXTRACTS IN DIABETIC WOUND HEALING

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ABSTRACT

Diabetic wound management has always been a challenge for health care providers and biomedical researchers. The present study was designed to investigate the wound healing potential of South Asian spice turmeric, which is rich in polyphenols, and honey in Alloxan-induced diabetic mouse model. Wound healing was improved significantly in topically applied turmeric, honey and insulin (as positive control) when compared with diabetic non-treated control, however highest wound healing was observed in the case of turmeric. Similarly, diabetic mice on oral turmeric showed improved healing as compared to the diabetic controls. Moreover, diabetic mice on oral turmeric showed enhanced glucose tolerance test. On the basis of above results we surmise that turmeric improves the healing activity in diabetic wounds by enhancing the glucose sensitivity. Our data emphasize the idea of using turmeric as ethnomedicine for diabetic wound healing.

Keywords: *Curcuma longa*; Pharmacology; Diabetes mellitus; Mice; Glucose Tolerance Test.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders largely characterized by higher level of blood glucose due to impaired metabolism of glucose. The resulting hyperglycemia results in plethora of complications including the inability to heal wounds (Gooyit *et al.*, 2014), which is one of the major complications in diabetic patients (Ferringer and Miller 2002). The delay in the search for appropriate treatment and the difficulty in managing diabetic complications hinder the healing process. The impaired wound healing in diabetes is associated with multitude of factors, resulting from the complications of the disease itself, including neuropathy, vascular disease, and foot deformities (Jude *et al.*, 1998; Jeffcoate and Harding, 2003). The impaired healing ultimately can result into loss of physical activity due to chronic wounds and diabetic foot ulcerations and life long disability due to limb amputation (Singh and Palmer, 2005).

Traditional plant-based medicine (phytochemicals) has long been used as a source of cure for various diseases all over the world including diabetes (Naseem *et al.*, 2016) and has also been used of for the management of wounds (Biswas and Mukherjee, 2003). Turmeric (*Curcuma longa*, belonging to the *Zingiberaceae* family), an extensively cultivated perennial herb in Southeast Asia, has long been used as a part of food due to its multiple health benefits (Chan *et*

al., 2009). Phytochemical profiling from the different types of turmeric extracts has indicated that the active constitutions of turmeric are various polyphenols like flavonoid and curcuminoids, whereas, 90% of curcuminoids content are contributed by *curcumin* only (Tayyem *et al.*, 2006). Various studies have attributed *curcumin* for anti-inflammatory, antibacterial, anti-fungal and for wound healing activities (Aggarwal and Sung, 2009). Moreover, extensive preclinical studies have explored curcumin's therapeutic potential against a wide range of human diseases (Aggarwal and Harikumar, 2009). Few studies have shown increased rate of wound healing has been observed in normal rats in response to application of different forms of turmeric (Emiroglu *et al.*, 2017; Kianvash *et al.*, 2017).

However, little information is available about the potential effectiveness of turmeric on diabetic wound healing; therefore, the aim of the present study is to evaluate the potential role of ethanolic extract of turmeric and honey on wound healing in experimentally induced diabetic mice.

MATERIALS AND METHODS

Chemicals and reagents: Different chemical and reagents like 100% Ethanol, methanol, Phosphoric acid, Folic-Ciocalteu (FC) reagent and Gallic acid were purchased from Sigma-Aldrich Corporation, USA and were of highest analytical grade.

Preparation of Pastes

Turmeric Paste: The fresh rhizomes of *Curcuma longa* L. were collected from the Vegetable Research Institute, Ayub Agricultural Research Institute (AARI), Faisalabad (Pakistan). The rhizome was weighed and dried at different drying temperature (20°C, 50°C, 70°C and Room Temperature i.e. 30 ± 2°C) in order to optimize the drying conditions. The dried samples were grounded into fine powder. For extraction purpose 1 g of dried turmeric powder was dissolve in 10 mL of 50% ethanol and methanolic solution separately. The solution was kept overnight at room temperature and filtered next day resulting into 5 mL of liquid extract. The total Phenolic Contents of each extract were determined spectrophotometrically using the Folin-Ciocalteu assay (Chaovanalikit and Wrolstad, 2004) in triplicate. In short, 200 µL of the crude extract was mixed with Folin-Ciocalteu reagent (200 µL). After 5 minutes at room temperature, 1 mL of 15 % sodium carbonate (w/v) was added. The samples were incubated at room temperature for 60 min. The absorbance was measured at 760 nm using a UV/Visible spectrophotometer (UV-1200, E-Chrom Tech CO., Ltd., Taipei, Taiwan). Gallic acid standards in the range of 50 – 500 mg/L were treated in a similar manner to generate a calibration curve ($R^2 = 0.9959$). The Total Phenolic Content (TPC) of the extracts was expressed as gallic acid equivalent (GAE) g/100 g dry weight. For preparation of turmeric paste liquid extract was mixed 1:1 v/w in petroleum jelly.

Insulin Paste: Insulin paste was used as positive control. For this, commercially available insulin [Humulin® R (U-100) by Eli Lilly Company, USA] was used in the concentration of 0.5 U/g of petroleum jelly.

Honey paste: For preparation of honey paste, naturally grown wild honey of honey bees (*Apis florea*) was mixed 1:1 w/w in petroleum jelly.

Animal Handling/Experimental Animal: Male albino mice of BALB/c strain were procured from University of Veterinary and Animal Sciences (UVAS), Lahore (Pakistan). Animals were of same age (approximately 8 weeks) and weighing about 30-35gms. They were housed in wooden cages (45 × 30 × 20 cm) lined with sterile husks that were cleaned every day. The mouse cages were placed in a controlled temperature room (22 ± 2°C) on a 12-hour light/dark cycle and were fed with standard mouse diet (Ho *et al.*, 2012) and water for regular intervals. Each mouse was placed in separate compartment within the cage after creating wounds. The mouse was acclimatized for 5-6 days before the initiation of experimental procedure. This study was performed after getting the approval by the Institutional Animal Ethics Committee of Directorate of Medical Sciences, Government College University Faisalabad (GCUF), Pakistan. All animals were treated in compliance with the

recommendations of the University's animal care committee and the principles of laboratory animal care.

Diabetes Induction in mice: Alloxan monohydrate (C₄H₂N₂O₄.H₂O) was purchased from AppliChem GmbH, Germany and used for diabetes induction. The mice were kept on 12h fasting period and Alloxan was administered to all the animals according to body weight (150 mg/kg of body weight) with slight modification as described in Takemoto *et al.*, 2016. Alloxan was dissolved in sodium chloride (0.9 %) solution and kept on ice to protect from degradation until injected. A single shoot of intra-peritoneal (IP) injection was administered and blood glucose level was monitored at regular interval by prickling a drop of blood from the tail vein of mouse using, SD Check™ blood glucose testing kit (SD Biosensor, Korea). Diabetic state of mice was confirmed in all animals after 9 days after multiple shoots of alloxan. The mice having blood glucose level more than 180 mg/dl were selected as diabetic and included in this study. Diabetes mellitus was not induced in non-diabetics control group.

Oral Turmeric suspension experiments: In another set of experiment two groups having 10 male diabetic mice were subjected to an oral turmeric powder suspension of three different concentrations each (5000 ppm in water) for 15 days (Treatment: Oral suspension). Two groups 10 male diabetic mice and 10 male non-diabetic mice were grouped as control with normal water. In these mice wounds were created as given above and Glucose tolerance test (GTT) performed as mentioned below, separately.

Wound Creation: Surgical procedures were performed under general anesthetized by intra-cutaneous injection of Ketamine HCL and Xylozine used in the concentration of 100 mg/kg and 7 mg/Kg of body weight respectively. The dorsal skin of the animals was shaved and cleaned with 70% ethanol. A full thick excision wounds of 5 mm in diameter were made on the dorsal skin (including epidermis, dermis, sub cutaneous fat and the underlying panniculus carnosus) using a sterile biopsy punch (Chen *et al.*, 2015). After recovery from anesthesia, mouse was housed individually in disinfected cages and treatments/pastes were applied topically twice a day for approximately 13-15 days or until the wound heals completely.

All the animals were divided into 5 groups of 6 in each. Group A was treated with insulin paste being the positive control. Next two groups B & C were treated with turmeric extract paste and honey paste respectively. Group D comprises of the diabetics control animals with no treatment. Finally, non-diabetic normal animals were grouped as Group E.

Photography of Wounds: Multi photograph of each wound of each animal was taken on daily basis after 24 h

interval starting post-wounding by Nikon DSL digital Camera with 18-55 mm zoom lens and subsequent measurements of wound areas from all the groups.

Measurement of Wound Size: The measurements of the edges of the wounds were done with computer software named Image J 1.48v NIH, USA. The mean diameter of the wound was calculated. The results of wound diameter measurements on various days (3, 6 and 9) were expressed as percentage wound healing. This data was utilized for making graphs by using the GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA). Percentage of wound contraction was calculated by using the following formula.

$$\% \text{ Wound healing} = \frac{0 \text{ day wound diameter} - \text{wound diameter on particular day}}{0 \text{ day wound diameter}} \times 100$$

Glucose Tolerance Test (GTT): The mice were kept on overnight fasting (approximately 16h) before the beginning of the experiment. The intra-peritoneal shoot of glucose (1 g/Kg of body weight) was injected to all the animals in both groups. However, the blood glucose was measured with glucometer at time point 0 min (before glucose shoot) and subsequent measurements were taken at 15, 30, 60, 90 and 120 minutes (Andrikopoulos *et al.*,

2008).

Statistical analysis: Results were analyzed using Minitab® v16 (LEAD Technologies, Inc) and expressed as mean \pm SEM. Difference among the group was compared by student's t-test (Anonymous, 2010).

RESULTS AND DISCUSSION

Effect of solvent and drying temperature on total phenol content: The nature of extracting solvent and method is crucial for the resulting extracts' yields for important bioactive compounds, as solvents system plays a vital role in extraction of maximum bioactive attributes by avoiding solvent that extract minimum compound (Ngo *et al.*, 2017). In this study, the difference of drying temperature and nature of organic solvent was tested for TPC. With the increase in drying temperature reduction in TPC was observed in ethanolic solvent with highest mean of 4.862 & lowest mean of 2.124 at 20°C & RT respectively. Whereas, mean TPC in methanolic solvent was lower (in range from 2.008 to 2.997) as compared to ethanol (Figure 1). So, ethanolic solvent extract at 20°C was selected to be used in the study owing to maximum yield of TPC.

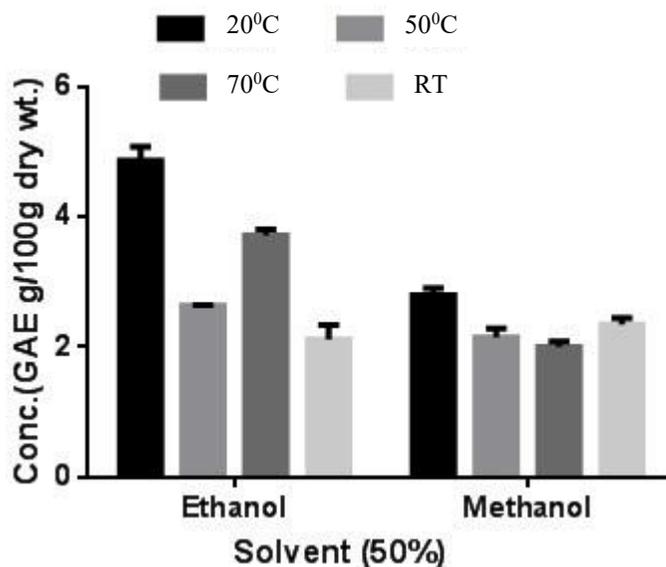


Figure 1: Total Phenolic Concentration of turmeric samples. Values are expressed as means \pm SEM (n = 3) for different extraction solvents and drying techniques, respectively. GAE = gallic acid equivalent.

Turmeric Accelerates Cutaneous Wound Healing in Diabetic Mice: During the process of wound healing granulation tissue formation is preceded by wound contraction leading to increased resistance, and fibroblasts differentiate into myofibroblasts which is responsible for early closure of wound (Desmoulière *et al.*, 1993). In this study the percent contraction of wound, the measure of the percentage of wound healing

depending upon the size of wound, was measured between different treated/untreated groups of mice (insulin and honey as positive controls) (Eyarefe *et al.*, 2014; Lima *et al.*, 2012). The percent (%) wound contraction of control vs. treated animals revealed significant difference was observed between the Turmeric treatment group and diabetic controls at 3, 6 and 9 days post wound creation (PWC) (Figure 2 and 3).

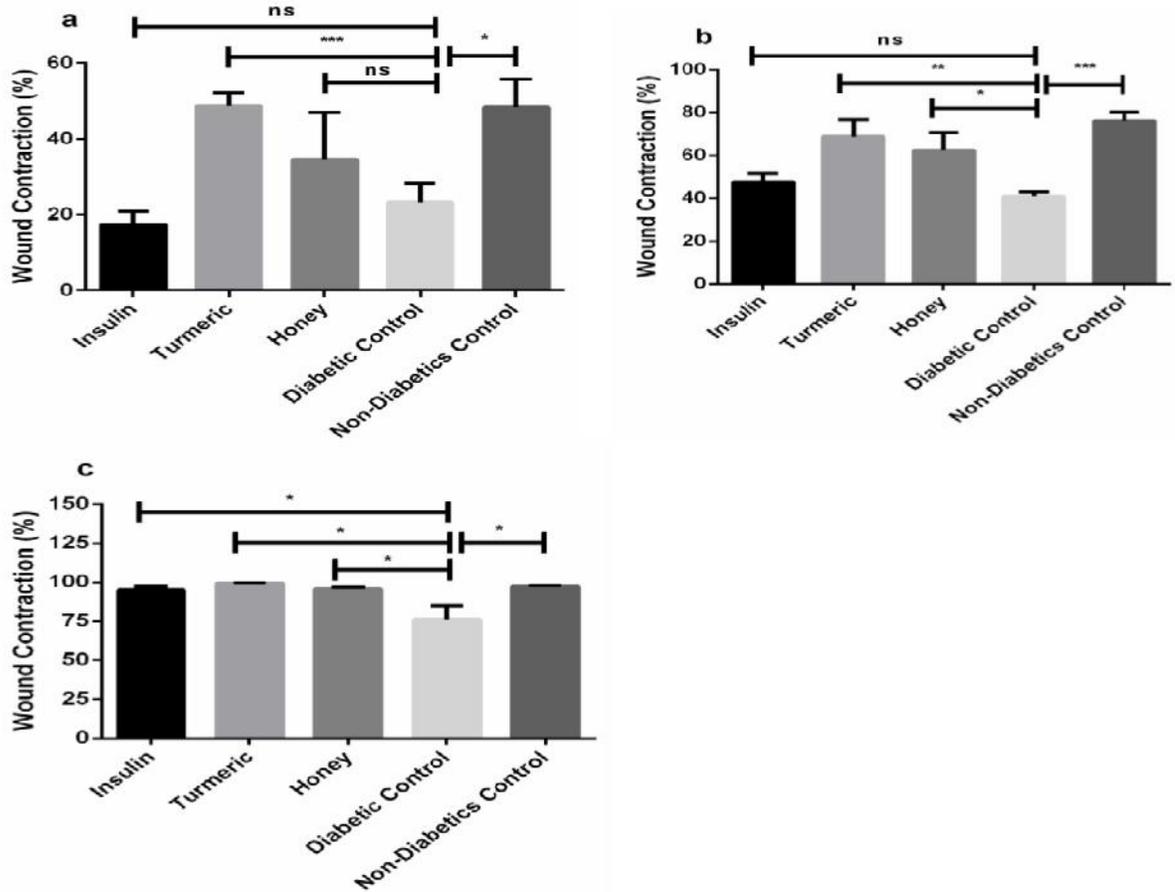


Figure 2: Effect of different treatments on wound contraction percentage at a) 3 days, b) 6 days, c) 9 days and post wound creation. Bars are expressed as Mean \pm SEM and *, ** and *** represent significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.005$ respectively from Diabetic control group.

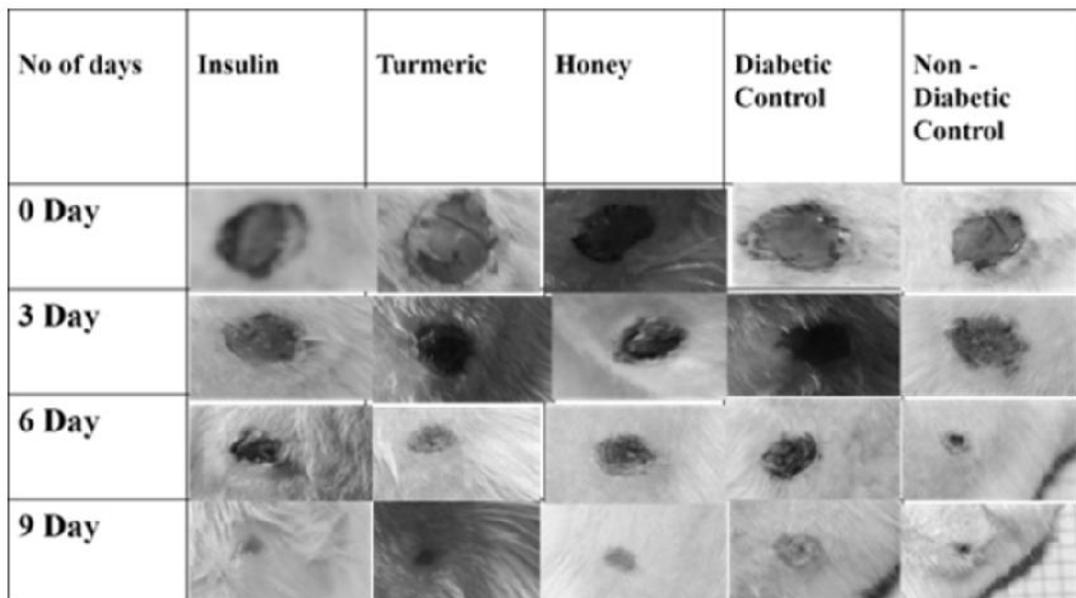


Figure 3: Effect of topical application of Turmeric paste on diabetic wound healing in mice at different day intervals.

At the same time, significant differences for healing response were observed between diabetic controls and non-diabetics controls at 3 and 9 days PWC with statistically pronounced effect at day 6 PWC showing that inflammatory as well as proliferative responses of wound healing were quite delayed in diabetic mice (Figure 2a, b & c). Interestingly, the significant differences between turmeric and insulin paste was observed only at 9 days PWC, attributing that topical application of insulin became more effective after prolong treatment. Additionally, honey paste (used as second positive control) proved better than insulin paste (Figure 2b & c). The experimental data shows an accelerated wound healing at 6th day PWC, depending upon the size of wound, in turmeric paste with 68.86%, honey paste with 62.45% and Insulin paste with 47.65% when compared with diabetic control (Figure 2b). Altogether data indicates that turmeric and/or honey independently increases healing activity as compared with diabetic control highlighting effectiveness of turmeric and/or honey as a wound healing ointment. Phenolic contents have been reported for antioxidant activities that might have resulted in stimulated wound healing process in mice treated with turmeric (Hanif *et al.*, 2016). Finally, almost complete wound healing has been achieved in all treatment groups except diabetic control group at 9th day PWC that was significant when compared with diabetic controls (Figure 2c).

Similar results and rate of healing were obtained (Data not shown) in the mice on oral turmeric (turmeric dissolved in drinking water) as compared to above-mentioned data on local application of turmeric. From this we hypothesized that some similar mechanisms enhancing the wound healing occurs acting both locally and systemically. The optimal activation of inflammatory mechanism is essential for proper wound healing. This enhanced healing response could be attributed to the modulation of inflammatory and proliferative responses (involving increase epithelialization, increase in

granulation tissue deposition, angiogenesis, etc.). Curcumin effectively scavenges the free radicals and reduces the oxidative stress, and decreased oxidative stress lowers the inflammatory response by inhibiting the transcription factor NF- κ B (Bharti *et al.*, 2003). Additionally, antimicrobial properties of curcumin, avoiding any wound infection, favours the better wound healing outcome observed in these experiments (Aggarwal and Sung, 2009).

Similar other studies involving herbal extracts from *Plantago major* and *Tecomastans* have been reported to improve wound healing (Mahmood and Phipps, 2006; Kameshwaran *et al.*, 2014). Moreover, positive effects of *curcumin* have also been reported in burn models of wound healing (Kulac *et al.*, 2013). Altogether, significant differences between the use of turmeric paste as compared to non-diabetic control indicates that turmeric is efficient for wound healing, and it might achieve better wound healing effect by modulating the better glycemic control (see below).

Turmeric induced enhanced glucose tolerance: The healing rate was enhanced in the diabetic mice on oral turmeric (turmeric dissolved in drinking water) as compared to diabetic controls. In this set of experiment with the oral use of turmeric, glucose clearance was enhanced in diabetic mouse as compared with the controls on normal drinking water. After intra-peritoneal injection of glucose, glucose levels significantly decreased in treatment groups as compared to controls, underscoring the effectiveness of oral turmeric in regulating glucose levels in the blood especially in diabetic conditions (Figure 4). We hypothesized that the different phytochemicals in turmeric modulate the metabolic machinery at cellular levels for metabolizing glucose. We speculate that polyphenolic agents in turmeric might be involved in the underlying mechanisms.

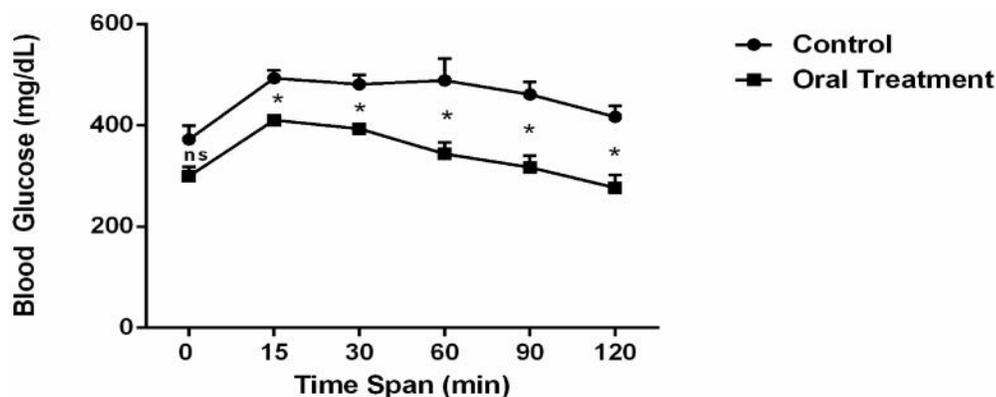


Figure 4: Glucose Tolerance Test (GTT) after 15 days of oral turmeric suspension treatment. Values are expressed as Mean \pm SEM. * shows significant differences between control group and 5000 ppm turmeric suspension treated group at each time interval.

Besides interleukin families including some chemokines and reactive oxygen species (ROS) are important due to their role in wound healing as well as in tissue repair process accompanying inflammatory reaction. Additionally, production of ROS further worsens the wound healing process, and polyphenolic compounds, as in turmeric, activate autophagic process, thereby scavenging the ROS and enhancing cellular repair and wound healing processes (Pietrocola *et al.*, 2012, Roberts and Sindhu, 2009). The role of the polyphenolic agents in modulating chemokines/interleukins and ROS during pathological/chronic wound healing, as in diabetes, needs particular more attention and warrants further experimentation for in depth studies.

Conclusion: Our study showed that wound healing in diabetic mice was significantly improved in topically applied turmeric when compared with topically applied honey and diabetic non-treated control, even better wound healing was observed with turmeric as compared to insulin treated mice as positive controls. On the basis of the results we surmise that turmeric, being rich in polyphenols, improves the healing activity in diabetic wounds by enhancing the glucose sensitivity. Our data underscores the importance of the use of ethnomedicine, such as turmeric, for diabetic wound healing though further studies are needed for their efficient use for better managing the diabetic wounds.

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