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The Journal of Phytopharmacology

(Pharmacognosy and phytomedicine Research)

Research Article

ISSN 2230-480X

JPHYTO 2016; 6(1): 1-10

January- March

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Pharmacological evaluation of wound healing activity of herbal based formulation “Amree Plus®” in diabetic rats

Sunita Thakur, Isha Dhamija*, Sandeep Kumar*, Anil Kumar Sharma, Santosh Kumar Verma

ABSTRACT

Delayed wound healing is producing high economic burden on the society and is generally associated with Diabetes mellitus or related complications thus needs safe treatment like herbal formulation. ‘Amree Plus® (an anti-diabetic ayurvedic formulation)’ was evaluated for wound healing potential in normal and diabetic rats. The effect of polyherbal formulation Amree plus ® on wound healing has been studied in diabetic and normal animals and compared with standard (Chymoral Forte) using excision wound model. Diabetes was induced by administration of single dose of Streptozotocin 55 mg/kg *i.p.* The biochemical parameters i.e. hydroxyl proline content, total protein content, glutathione and superoxide dismutase (SOD) were also estimated. Rats treated with Amree Plus® at both doses i.e. 1200 and 5400 mg/kg dose per day had significantly high wound contraction both in diabetic and normal rats (88.91 & 95.05 %, respectively) in comparison to respective control. Hydroxyproline content of the granulation tissue and anti-oxidants (glutathione and SOD) level were also showing same pattern in Amree Plus® 5400 mg/kg treatment group with respect to controls. Wound contraction and other biochemical indicators results with animals Amree Plus® (1200 mg/kg) treated were found to be statistically equivalent to standard chymoral forte dose (36 kAU/kg) while Amree Plus® 5400 mg/kg dose showed statistically higher results than standard. Amree Plus®, an established antidiabetic formulation, was also showing healing of the wound in normal animals along with diabetic animals. So this finding should be confirmed further for more precision with large number of animals and small progression of doses.

Keywords: Wound healing, Amree Plus®, Chymoral forte, Anti-oxidants, Hydroxy proline.

INTRODUCTION

Diabetes mellitus, which is a morbid condition associated with various connective tissue (mainly collagen tissue) abnormalities, is generally associated with delayed wound healing. Delayed wound healing is producing high economic burden on the society. Thus research is required to develop new and effective treatment strategies to deal with this emerging issue i.e. delayed wound healing. Diabetic foot ulcer is severe most case of delayed wound healing which afflicts 15% of diabetic patients and among these 84% undergo lower-leg amputations.^[1] Delay in wound healing, specifically to diabetic patients, is due to ill function/metabolic behavior/chemotaxis of leucocytes resulting in non availability of neutrophils and macrophages to the wound.^[2] Basic principle of wound healing is to reduce tissue damage by providing adequate blood supply, oxygenation, proper nutrition and moist environment for healing of the wound^[3] It involves the organized and coordinated interaction between extracellular matrix, different types of cells (leukocytes, fibroblasts etc.) and various growth factors such as platelet derived growth factor, epidermal growth factor, fibroblast growth factor etc.^[4] Herbal treatment has been targeted in the study, as of their greater demand in market now-a-days. As there are many plants as such or their fruit/seed/leaves etc or their extracts/ reported to have efficacy in various ailments, like *Tamarindus indica* in depression and anxiety^[5], *sesamum indicum* in anxiety^[6], *Premna herbacea* in tumour^[7], *Mellilotus officinalis* in wound healing^[8] and a polyherbal formulation ‘Neeri®’, used in urolithiasis^[9]. Moreover herbal formulations has the advantage of being given orally, which most convenient way of delivery of drug, as the multiconstituent combination in the formulation act as a stabilizer in *in vivo* system to the active constituent while many isolated compounds has the issue of instability and many a times given parentally. ‘Amree Plus®’, a poly-herbal formulation already available in the market, is effective in controlling blood glucose by delaying glucose absorption and other complications related to diabetes. Apart from anti-diabetic action, it also provides toning effect, nutrient supplementation, anti-oxidant, immuno-modulator, anti-ageing, cardio-protection and renal protection. Amree plus® exert its effect by activating islets of pancreas and mimicking insulin effect.^[10] Chemical examination of ‘Amree Plus®’ revealed the presence of flavonoids, tri-terpenoids, saponins,

tannins, steroids, proteins, amino acids etc. It consists of many plants reported to have wound healing activity such as *Gymnema sylvestre* (Gudmar), *Pterocarpus marsupium* (Vijaysaar) ^[11], *Eclipta alba* (Bhringraj) ^[12], *Azadirachta indica* (Neem patr) ^[13], *Coccinia indica* (Kundru) ^[14], *Nigella sativa* (Kalonji) ^[15], *Tinospora cordifolia* (Giloe) ^[13], *Aegle marmelos* (Bilv patr) ^[16] etc. These plants contains secondary metabolites that promotes wound healing through different components such as saponins (anti-oxidant and anti-microbial activity), tannins and flavonoids (astringent, anti-microbial property and free radical scavengers), sterols & poly phenols (free radical scavengers, anti-oxidant and reduce lipid peroxidation), tri-terpenoids (promotes wound contraction and rate of epithelialization).^[17]

This formulation shows good antidiabetic activity and has herbal constituents active as wound healer thus it may heal wound or lessen the delayed wound healing in diabetics. But this formulation has not investigated yet for wound healing activity, either in normal or in diabetic animals. The present study was undertaken to evaluate wound healing potential of anti-diabetic formulation Amree Plus® in diabetic and non-diabetic rats based on preliminary study and folkloric information.

MATERIAL AND METHODS

Materials and Drugs

Amree Plus®, a polyherbal anti-diabetic formulation (Aimil Pharmaceuticals Ltd., India), was used as test drug. Chymoral forte (Elder Pharmaceutical Pvt. Ltd.) a wound healing and anti-inflammatory enzyme comprising of a purified mixture of proteolytic enzymes trypsin and chymotrypsin (in ratio of approximately 6 to 1 and provides enzyme activity equivalent to 100 k Armour units (AU)/tablet) was used as reference drug. Streptozotocin (STZ), Chloramine T, phenol reagent, 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB) or Ellman's reagent, nitroblue tetrazolium (NBT), phenazine

methosulphate (PMS), nicotinamide adenine dinucleotide (NADH) were procured from Sigma Aldrich. Rest of chemicals and reagents are of analytical grade.

Preliminary Phytochemical screening

Preliminary phytochemical screening of Amree Plus® was carried out to detect the presence of glycosides, flavonoids, alkaloids, steroids, tannins, proteins, amino acid, carbohydrates as per the standard methods given in literature.^[9,18]

Experimental protocol

Procurement and selection of animals

Normal and healthy adult albino rats (male) of Wistar strain (3-5 months), weighing between 200-250 g (procured from Panacea Biotech Ltd. Lalru, Punjab), were used for the experimental study. All the animals were fed on a standard rat feed and water *ad libitum* throughout the experiment. Animal studies were conducted according to the Institutional Animals Ethics Committee (IAEC) regulations approved by Committee for the Purpose of Control and Supervision of Experiments on Animals [Protocol no: IAEC-CTIPS/2014/VI/0037(PCL-M)]. The animals were housed individually in polypropylene cages with clean and sterile paddy husk bedding, under controlled temperature conditions (24 ± 2°C) as well as humidity (60±5%) and 12 h light-dark cycles.

Experimental design

Grouping of animals

Total of 48 rats were used and randomly divided into eight groups, each group containing six rats (Table 1).

Table 1: Grouping of animals

Groups	Name	Treatment (n=6)
Group I	Normal control (NC)	Normal rats with wound, given saline 10 mL/kg
Group II	Diabetic control or negative control (DC)	Diabetic rats (STZ induced) with wound, received standard pellet diet & water <i>ad libitum</i>
Group III	Normal standard i.e. Chymoral forte group (NCF)	Normal rats with wound were treated with reference standard Chymoral Forte (36k AU/kg of rat body) <i>p.o.</i> for 21 days
Group IV	Diabetic standard i.e. Chymoral forte group (DCF)	Diabetic rats with wound were treated with reference standard Chymoral Forte (36k AU/kg of rat body), <i>p.o.</i> for 21 days
Group V	Normal test i.e. Amree Plus (1200 mg/kg of rat body) group (NAP1)	Normal rats with wound were treated with Amree Plus at minimum (1200 mg/kg of rat body) dose, <i>p.o.</i> for 21 days
Group VI	Normal test i.e. Amree Plus (5400 mg/kg of rat body) group (NAP2)	Normal rats with wound were treated with Amree Plus at maximum (5400 mg/kg of rat body) dose, <i>p.o.</i> for 21 days
Group VII	Diabetic test i.e. Amree Plus (1200 mg/kg of rat body) group (DAP1)	Diabetic rats with wound were treated with Amree Plus at a minimum (1200 mg/kg of rat body) dose, <i>p.o.</i> for 21 days
Group VIII	Diabetic test (5400 mg/kg of rat body) group (DAP2)	Diabetic rats with wound were treated with Amree Plus at a maximum (5400 mg/kg of rat body) dose, <i>p.o.</i> for 21 days

Induction of diabetes

The protocol for induction of diabetes has been observed from literature and was used with little modifications. The animals of group II, IV, VII and VIII were weighed and fasted overnight before administration of Streptozotocin. Diabetes was induced by single dose of Streptozotocin (STZ, 55 mg/kg, *i.p.*) in ice cold 0.1M sodium citrate buffer (pH 4.5, freshly prepared), injected intraperitoneally and 5% glucose solution was supplied to avoid sudden hypoglycaemia post-injection. Then fasting blood glucose was measured on 7th day after STZ injection by glucometer to ensure the diabetes induction. Rats with blood glucose levels >250 mg/dl are considered as diabetic and included in the study.^[19]

Creation of wounds

Excision wound model was used in both normal and diabetic animals for this study. Method in brief is as follows, the back of the rats were depilated under ketamine injection (50 mg/kg, *ip*) anesthesia and surgical area was disinfected with the alcohol-iodine solution. Then circular open wound of full thickness was made on the predetermined area (approximately 1.5 cm) outlined with the marker. Wounds were left undressed to the open environment and each animal was kept individually in separate cages. Wounds were traced on 1 mm² graph paper on the zero day (day of wound creation) and subsequently after a gap of days i.e. day 3rd, 6th, 9th and so on till 21st day of the study after diabetes induction.^[16, 20]

Treatment

The treatment of standard i.e. Chymoral forte (36 k AU/kg), test 1 i.e. Amree Plus® 1200 mg/kg and test 2 i.e. Amree Plus® 5400 mg/kg was given in respective group of animals (Table 1) daily for 21 days. Diabetes was induced 7 days (time of diabetic model preparation) prior to the treatment/wound model preparation.

Percent wound contraction was calculated by using following formula.

$$\% \text{ Wound contraction} = \frac{\text{Wound size at day 0} - \text{Wound size at specific day}}{\text{Wound size at day 0}} \times 100$$

Estimation of hydroxy proline content

On 21st post operative day, granulation tissue of the animals from each group is collected and weighed. Then the collected granulation tissue was dried (60-70 °C) in an oven for 24 h and the dry tissue weight was again noted. Dried tissue was hydrolysed in 10 ml of 6 N HCl for 4 h at 130 °C and hydrolysate was cooled and neutralized to pH 7 by using 10 N NaOH. Then the neutralized hydrolysate was subjected to Chloramine-T oxidation (20 min). This reaction is terminated by addition of 0.4 M perchloric acid and developed colour with Ehrlich reagent at 60 °C. Then the absorbance was read at 557 nm in UV Spectrophotometer after 1 h^[21]

Assessment of total protein

0.05 ml of granulation tissue homogenate was dissolved in 1 ml of 0.1 N NaOH. From this sample 0.4 ml is taken into another test tube. To this mixture 4 ml of alkaline reagent was added and kept for 10 minutes. Then 0.4 ml of the phenol reagent was added and again it

was allowed to stand for 10 minutes to develop colour. Readings were taken against the blank prepared with water at 610 nm.^[22,23]

Assessment of reduced glutathione (GSH)

To 1 ml of tissue homogenate (prepared in 100 mg/mL in phosphate buffer) 0.8 ml distilled water and 0.2 ml TCA (50 %) was added and shaken well (10-15 minutes). Then the mixture is centrifuged at 3000 rpm for 15 minutes. To the 0.6 ml of supernatant 0.8 ml of Tris buffer (0.4 M, pH 8.9) and 20 µl of 0.1 M DTNB in absolute methanol was added and shaken well. After that 40 µl DTNB is added to the reaction mixture and absorbance is measured at 412 nm within 5 min.^[7,21]

Assessment of superoxide dismutase (SOD)

To 0.2 ml of tissue homogenate 0.6 ml of sodium pyrophosphate buffer (0.052 M, pH 8.3), 50 µl of PMS (186 µM), 150 µl of NBT (300 µM) and 0.4 ml of distilled water was added and reaction was started by the addition of 0.1 ml of NADH (780 µM) to this sample. This sample was incubated for 60 seconds and then 0.5 ml of glacial acetic acid was added to stop the reaction. To this reaction mixture 2 ml of n-butanol was added and stirred vigorously and allowed to stand for 10 min. After that this mixture was centrifuged for 10 minutes (3000 rpm) and then the butanol layer containing formazan was separated and absorbance was measured at 560 nm wavelength in spectrophotometer against n-butanol.^[16]

Statistical analysis

All results were expressed as mean ± standard error (SEM). Data was analyzed by using One way ANOVA followed by Tukey's multiple comparison tests using GraphPad InStat. P<0.05 was considered as statistically significant.

RESULTS

Phytochemical Screening

On preliminary phytochemical screening, Amree Plus® showed the presence of secondary metabolites shown in Table 2.

% Wound Contraction

In order to find the effect of Amree Plus® on % wound contraction of normal as well as diabetic rats, wounds were created and then animals were treated with standard and AP treatments (Figure 1). Amree plus® showed dose and time dependent increase in percent wound contraction in both diabetic and normal rats. Wound contraction measured on "15th day" was compared among all groups of animals and results are shown in Figure 2. Reference drug i.e. Chymoral Forte (36 k AU/kg) treated animals in both normal (NCF) and diabetic (DCF) animals showed significant (p<0.05) increase in wound contraction i.e. 94.76 % and 71.06 % respectively when compared to non diabetic control (NC) and diabetic control (DC) animals. Moreover, the fastest wound contraction was observed in NCF group i.e. reference drug in normal animals (not diabetic). Amree Plus® at both doses (1200 mg/kg & 5400 mg/kg) also showed significant (p<0.05) increase in wound closure i.e. 75.33 % & 65.15 % for NAP1 & DAP1 and 88.91 % & 95.05 % for NAP2 & DAP2 respectively, when compared to NC and DC animals.

Table 2: Phytochemical constituents of “Amree plus”

S. No.	Phyto-constituents	Test	Result
1.	Steroids	a) Salkowski reaction	Present
		b) Liebermann’s burchard reaction	Present
2.	Glycoside	a) Keller Killani test	Present
		b) Borntrager’s test	Present
		c) Legal test	Present
3.	Carbohydrate	a) Fehling’s test	Present
		b) Benedict’s test	Present
4.	Alkaloids	a) Mayer’s test	Present
		b) Murexide test	Present
5.	Tannins	a) 5% FeCl ₃	Present
		b) Dilute HNO ₃	Present
6.	Proteins	a) Millon’s test	Present
		b) Xanthoprotein test	Present
7.	Amino acid	a) Ninhydrin test	Present
		b) Millon’s reagent test	Present
8.	Flavonoids	a) Lead acetate	Present
		b) Sodium hydroxide	Present

It was also found that animals treated with Amree Plus® at lower dose (1200 mg/kg) showed statistically equivalent results as compared to animals treated with CF in diabetic rats, exhibiting equipotency of Amree Plus® with Chymoral forte (observe the absence of “###” in diabetic AP1 group in Figure 2). Higher dose of Amree plus® (5400 mg/kg) showed significant difference in diabetic animals as compared to Chymoral forte treated diabetic animals, revealing that Amree Plus® is more active in diabetic animals than standard drug (observe the high mean value and presence of “###” in diabetic AP2 group in Figure 2). Moreover, Amree Plus® at 5400 mg/kg proved itself more promising as compared to lower dose of Amree plus® (1200 mg/kg) in normal as well as diabetic group (observe the presence of “&” and “&&” in normal and diabetic AP2 groups, respectively in Figure 2).

Effect of ‘Amree Plus®’ on hydroxy proline content

Significant (p<0.05) enhancement was observed in hydroxy proline content in wound tissues of the normal and diabetic rats, treated with Amree Plus® at both doses i.e. 1200 mg/kg and 5400 mg/kg i.e. 49.66 & 44.83 mg/g of dry tissue weight and 52.16 & 53.66 mg/g of dry tissue weight respectively, when compared with control group of normal and diabetic rats respectively. Furthermore, Amree Plus® at dose 5400 mg/kg showed comparable results to that of reference drug i.e. Chymoral Forte at dose 36 k AU/kg (Figure 3). Non-significant difference between reference drug CF and Amree plus® (at both doses) and in both the conditions i.e. normal and diabetic can be observed visually by the absence of “#”, “##”, in AP1 and AP2 groups in Figure 3 proves equiefficacy of Amree Plus® and reference drug.

Effect of ‘Amree Plus®’ on total protein content

On 21st day of the treatment period, the protein content of the animals treated with Amree Plus® (5400 mg/kg) and reference drug i.e. Chymoral Forte (36k AU/kg) was estimated. Protein content was found to be significantly (p<0.05) increased in all groups i.e NCF, DCF, NAP1, DAP1, NAP2 and DAP2 in comparison to the normal and diabetic rats respectively. Amree Plus® at high dose i.e. 5400 mg/kg was showing higher mean of protein content in diabetic groups

than that of standard i.e DCF (but was statistically equivalent, observe the absence of ## at AP2 columns), while normal groups its mean was lower than NCF but again statistically equivalent Figure 4.

Effect of ‘Amree Plus®’ on super oxide dismutase (SOD)

The level of super oxide dismutase (SOD), a free radical scavenger, was also found to be increased in diabetic and normal rats treated with Amree Plus®. Amree Plus® at dose 5400 mg/kg and Chymoral Forte (36k AU/kg) showed significant (p<0.05) improvement in the level of SOD, when compared to the control animals i.e. normal and diabetic control. Amree plus® at dose 1200 mg/kg was found to be significant (p<0.05) in normal animals but was insignificant (p>0.05) in diabetic animals when compared to the normal and diabetic control (Figure 5). AP2 i.e. Amree Plus® at 5400 mg/kg and reference drug showed statistically equal levels of SOD, demonstrating equivalent effects in both normal as well as diabetic animals. The higher dose of Amree Plus® i.e. 5400 mg/kg (DAP2) was showing significantly high levels of SOD (34.5 µg/mg of dry tissue weight) than lower dose Amree Plus® i.e. 1200 mg/kg (DAP1) (19.83 µg/mg of dry tissue weight) in diabetic animals. (Observe the presence of ‘&&’ in Figure 5)

Effect of ‘Amree Plus®’ on reduced glutathione (GSH)

The levels of the GSH were found to be increased significantly (p<0.05) in diabetic animals treated with Amree Plus® at both doses i.e. 1200 mg/kg and 5400 mg/kg. Animals treated with Amree Plus® at dose of 5400 mg/kg showed statistically equivalent results to that of animals treated with Chymoral Forte (36k AU/kg) in both normal and diabetic animals (Figure 6).

































	Day 0	Day 6	Day 12	Day 18
NC				
% WC	0	21.21	45.45	91.51
DC				
% WC	0	10.05	28.99	69.98
NCF				
% WC	0	29.03	67.75	100
DCF				
% WC	0	33.35	49.21	84.81
NAP1				
% WC	0	21.28	52.02	93.42
DAP1				
% WC	0	15.12	42.77	82.08
NAP2				
% WC	0	39.29	51.65	82.54
DAP2				
% WC	0	26.85	88.57	100

Figure 1: Wound and its contraction with the progression of time in different treatments

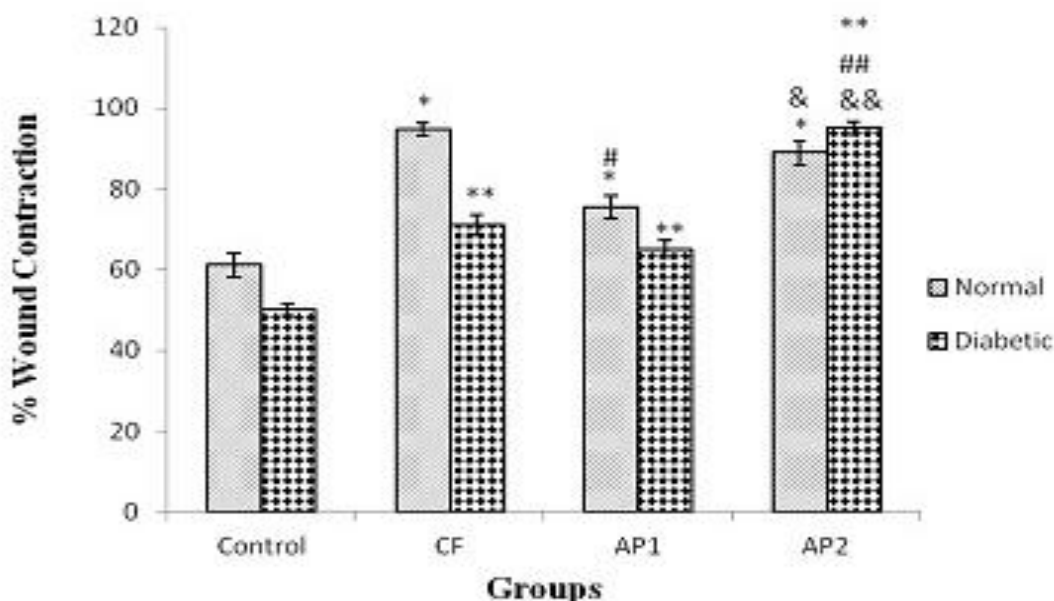


Figure 2: % wound contraction on 15th day

Normal control (NC), Normal chymoral forte (NCF): Chymoral forte at dose 36k AU/kg, p.o administered to normal rats; Normal AP1 (NAP1): Amree Plus® at dose 1200 mg/kg, p.o administered to normal rats; Normal AP2 (NAP2): Amree Plus® at dose 5400 mg/kg, p.o administered to normal rats; Diabetic control (DC), Diabetic CF (DCF): Chymoral forte (36k AU/kg, p.o.) administered to diabetic rats, Diabetic AP1 (DAP1): Amree Plus® (1200 mg/kg, p.o.) administered to diabetic rats, Diabetic AP2 (DAP2): Amree Plus® (5400 mg/kg, p.o.) administered to diabetic rats.

All values are expressed as mean±SEM (n=6); * represents significance (p<0.05) as compared to control, ** represents significance (p<0.05) as compared to diabetic control, # represents significance (p<0.05) as compared to normal CF, ## represents significance (p<0.05) as compared to diabetic CF, & represents significance (p<0.05) as compared to normal AP1, && represents significance (p<0.05) as compared to diabetic AP1. Analyzed by one way ANOVA followed by Tukey's tes.

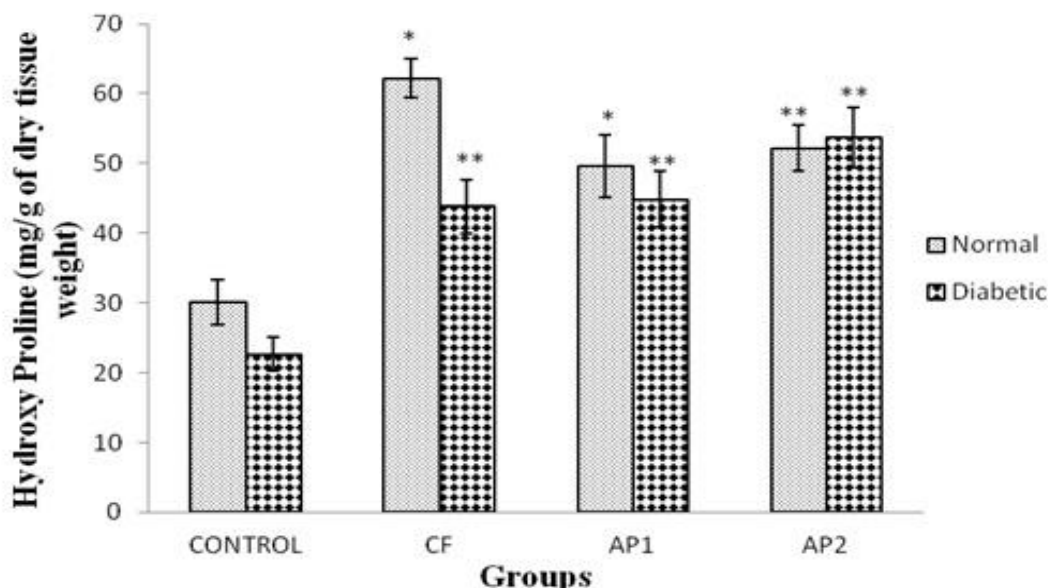


Figure 3: Effect of 'Amree Plus®' on hydroxy proline content in tissue homogenate

Normal control (NC), Normal chymoral forte (NCF): Chymoral forte at dose 36k AU/kg, p.o administered to normal rats; Normal AP1 (NAP1): Amree Plus® at dose 1200 mg/kg, p.o administered to normal rats; Normal AP2 (NAP2): Amree Plus® at dose 5400 mg/kg, p.o administered to normal rats; Diabetic control (DC), Diabetic CF (DCF): Chymoral forte (36k AU/kg, p.o.) administered to diabetic rats, Diabetic AP1 (DAP1): Amree Plus® (1200 mg/kg, p.o.) administered to diabetic rats, Diabetic AP2 (DAP2): Amree Plus® (5400 mg/kg, p.o.) administered to diabetic rats. All the treatments were administered for 21 successive days.

All values are expressed as mean±SEM (n=6); * represents significance (p<0.05) as compared to control, ** represents significance (p<0.05) as compared to diabetic control, # represents significance (p<0.05) as compared to normal CF, Analyzed by one way ANOVA followed by Tukey's test.

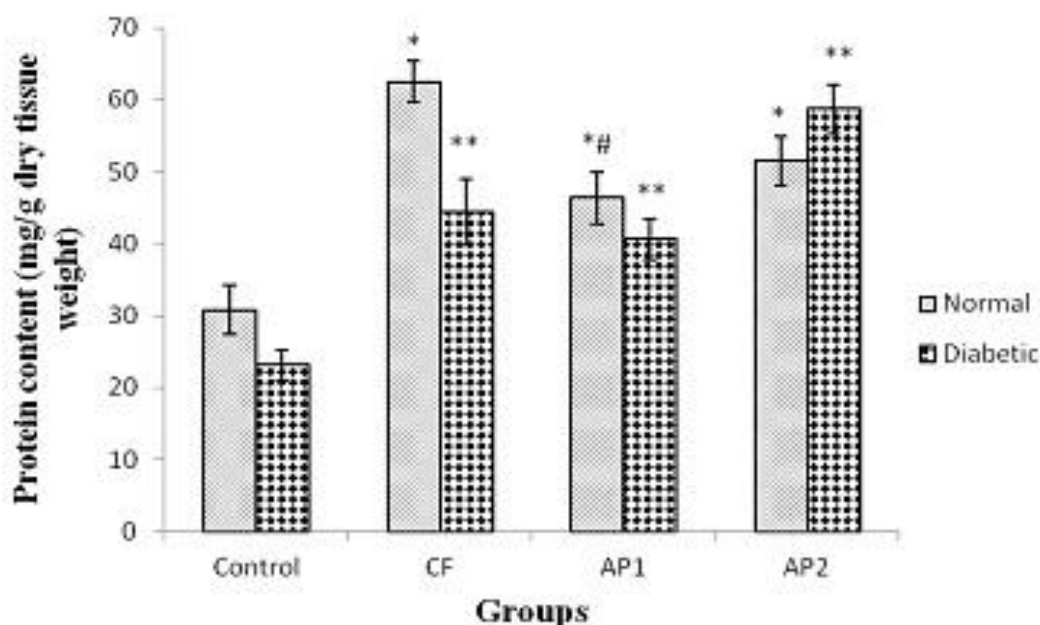


Figure 4: Effect of 'Amree Plus®' on total protein content in tissue homogenate

Normal control (NC), Normal chymoral forte (NCF): Chymoral forte at dose 36k AU/kg, p.o administered to normal rats; Normal API (NAP1): Amree Plus® at dose 1200 mg/kg, p.o administered to normal rats; Normal AP2 (NAP2): Amree Plus® at dose 5400 mg/kg, p.o administered to normal rats; Diabetic control (DC), Diabetic CF (DCF): Chymoral forte (36k AU/kg, p.o.) administered to diabetic rats, Diabetic API (DAP1): Amree Plus® (1200 mg/kg, p.o.) administered to diabetic rats, Diabetic AP2 (DAP2): Amree Plus® (5400 mg/kg, p.o.) administered to diabetic rats. All the treatments were administered for 21 successive days

All values are expressed as mean±SEM (n=6); * represents significance (p<0.05) as compared to control, ** represents significance (p<0.05) as compared to diabetic control, # represents significance (p<0.05) as compared to normal CF, Analyzed by one way ANOVA followed by Tukey's test.

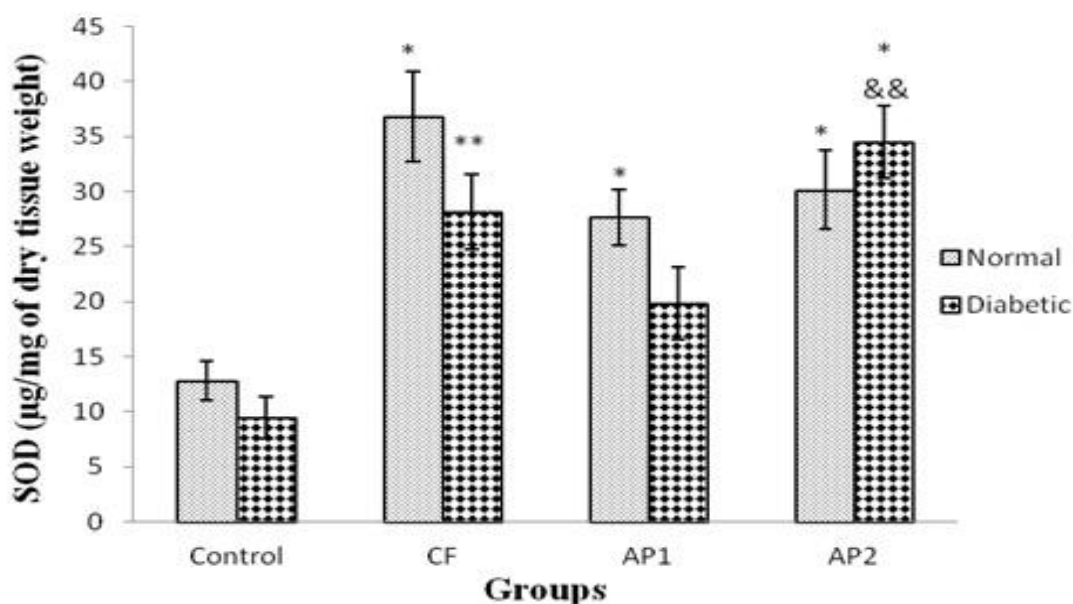


Figure 5: Effect of 'Amree Plus®' on super oxide dismutase (SOD) in tissue homogenate

Normal control (NC), Normal chymoral forte (NCF): Chymoral forte at dose 36k AU/kg, p.o administered to normal rats; Normal API (NAP1): Amree Plus® at dose 1200 mg/kg, p.o administered to normal rats; Normal AP2 (NAP2): Amree Plus® at dose 5400 mg/kg, p.o administered to normal rats; Diabetic control (DC), Diabetic CF (DCF): Chymoral forte (36k AU/kg, p.o.) administered to diabetic rats, Diabetic API (DAP1): Amree Plus® (1200 mg/kg, p.o.) administered to diabetic rats, Diabetic AP2 (DAP2): Amree Plus® (5400 mg/kg, p.o.) administered to diabetic rats. All the treatments were administered for 21 successive days.

All values are expressed as mean±SEM (n=6); * represents significance (p<0.05) as compared to control, ** represents significance (p<0.05) as compared to diabetic control, && represents significance (p<0.05) as compared to diabetic API, Analyzed by one way ANOVA followed by Tukey's test.

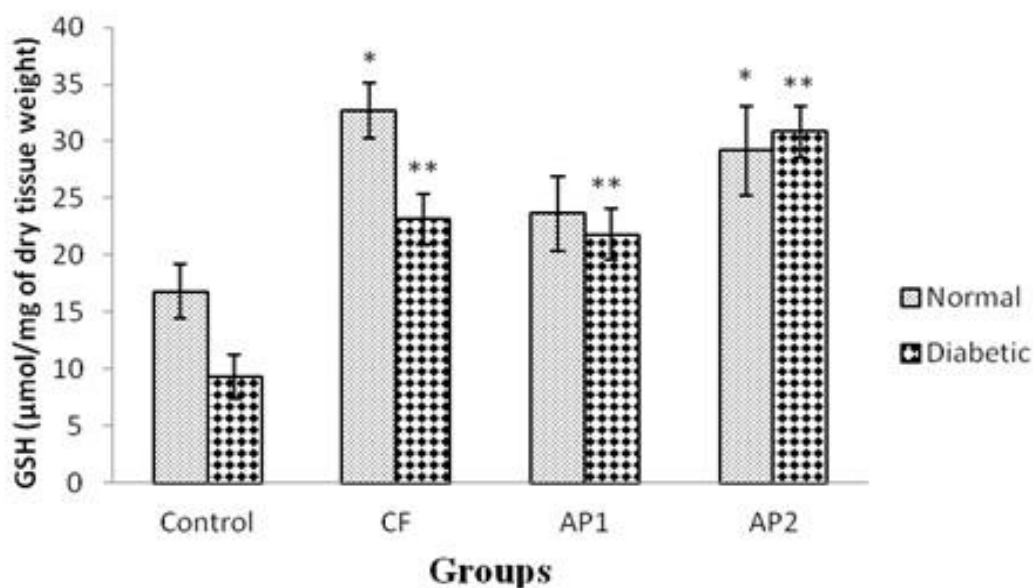


Figure 6: Effect of ‘Amree Plus®’ on reduced glutathione (GSH) in tissue homogenate

Normal control (NC), Normal chymoral forte (NCF): Chymoral forte at dose 36k AU/kg, p.o administered to normal rats; Normal API (NAP1): Amree Plus® at dose 1200 mg/kg, p.o administered to normal rats; Normal AP2 (NAP2): Amree Plus® at dose 5400 mg/kg, p.o administered to normal rats; Diabetic control (DC), Diabetic CF (DCF): Chymoral forte (36k AU/kg, p.o.) administered to diabetic rats, Diabetic API (DAP1): Amree Plus® (1200 mg/kg, p.o.) administered to diabetic rats, Diabetic AP2 (DAP2): Amree Plus® (5400 mg/kg, p.o.) administered to diabetic rats. All the treatments were administered for 21 successive days.

All values are expressed as mean±SEM (n=6); * represents significance (p<0.05) as compared to control, ** represents significance (p<0.05) as compared to diabetic control, Analyzed by one way ANOVA followed by Tukey’s test.

DISCUSSION

Wound healing is a complex and natural response of the body that results in the achievement of the restoration of the normal physiological function and integrity of the injured/damaged tissues and also produces several extracellular matrix proteins and growth factors [24]. Chymoral forte which is used as reference drug in this study is known to have the efficacy of diminishing the increased levels of cytokines and improving the wound re-epithelialization and healing of injured site.[25] In the present study, Amree Plus® at both doses (1200 mg/kg, 5400 mg/kg) showed significant increase in % contraction of wounds in normal as well as diabetic rats as compared to the normal control and diabetic control respectively. This enhanced wound contraction can be credited to enhanced contractile property of myofibroblasts or increased number of myofibroblasts recruited at the site of injury.[26] Hermes et al also reported that *Ipomoea batatas* (sweet potato) significantly increased the wound healing properties. Ointment formulation of sweet potato at concentration 2.5% was found to improve wound healing, which was confirmed by metaphase stimulation in cells and re-epithelialisation of tissue, within four hours of treatment. The wound healing activity of this plant might be possessed due to the carotenoids and poly-phenols present in the tubers of the plant.[27] While one research group isolated one fraction of active compound hyperoside from *Rubrus sanctus* has shown wound healing properties with *in-vitro* estimators viz. hyaluronidase, collagenase and elastase inhibitors.[28]

‘Amree Plus®’ is a poly herbal formulation consisting of many plants that contains various secondary metabolites that promotes wound healing through different components such as saponins (anti-oxidant and anti-microbial activity), tannins and flavonoids (astringent, anti-microbial property and free radical scavengers), sterols & poly

phenols (free radical scavengers, anti-oxidant and reduce lipid peroxidation), tri-terpenoids (promotes wound contraction and rate of epithelialization).[17] *Coccinia indica* (Kundru) is one of the components of Amree Plus®, which is proved to have anti-diabetic [29] and wound healing activity [14]. Wound healing activity of this plant is exhibited due to the presence of various active constituents in it i.e. terpenes, alkaloids, flavonoids, terpenoids, tannins etc.[30] Flavonoids and their derivatives promote wound healing by reducing lipid per-oxidation, thereby improving vascularity and preventing or slowing down the process of cell necrosis. Thus increased level of flavonoids leads to increase in the viability and strength of collagen fibrils along with improved blood circulation.[31] Lodhi and Singhai demonstrated that flavonoids like leuteolin potentially enhance the wound healing in diabetic conditions due to free radical scavenging activity.[21] Therefore, the wound healing activity of the Amree Plus® may be due to the additive effect of these phytoconstituents present in this formulation.

Hydroxy proline is an amino acid and is the major constituent of the collagen.[21] The level of hydroxyproline gets decreased at the site of the wound. Wound healing mainly depends upon the simultaneous formation and maturation of the collagen, as collagen is the major component of the wound tissues. In diabetic patients, collagen content of the skin get reduced due to decreased formation and increased degradation of the newly synthesized collagen.[11] Collagen is the major protein of the extracellular matrix and its breakdown liberates free hydroxy proline which gives the measurement of collagen turnover.[32] Hydroxy proline content in wounds treated with Amree Plus® at dose 5400 mg/kg found to be significantly high in normal and diabetic rats when compared with the normal and diabetic control respectively. Gowda et al demonstrated that *Michelia champaca* flower extract significantly (p<0.05) increase the hydroxy proline

content in non-diabetic rats as compared to the diabetic rats.^[33] Total protein content was also found to be significantly ($p<0.05$) increased in Amree Plus® treated animals as compared to normal and diabetic control as collagen content get increased.

Reactive Oxygen Species (ROS) plays a vital role in process of wound healing. These include oxygen-derived radical as well as non-radical oxidants, which are often termed oxidants or free radicals. ROS acts as cellular messengers that drive numerous biological pathways of wound healing such as at optimum micromolar concentration, hydrogen peroxide (H_2O_2) can promote vascular endothelial growth factor (VEGF) expression in keratinocytes and increase the wound healing rate. At normal concentration, they are always beneficial to body, but when their levels get elevated, they induce severe tissue damage and even lead to neoplastic transformation.^[34] Overproduction of ROS causes chemical modification in the cells by causing damage to proteins, lipids, carbohydrates and nucleotides, resulting in oxidative stress, cytotoxicity leading to delayed wound healing.^[35]

During the inflammation phase of wound healing, inflammatory cells get increased at the site of the injury and produce huge amount of the ROS, affecting the wound.^[36] Diabetes results in oxidative stress, which leads to the production of free radicals that in turn cause tissue damage and delayed healing, so the administration of antioxidants promotes the healing of wounds.^[37] The decreased level of antioxidants leads to the increased level of oxidative stress. Superoxide gets rapidly converted to H_2O_2 in the presence SOD, H_2O_2 release may promote the formation of other oxidants which may be more stable; suggesting that the level of the oxidants get increased at the site of wound which leads to the delayed wound healing. Antioxidants therefore, enhance the wound healing rate by decreasing the damage caused by free radicals.^[38] The study showed that the level of SOD in the tissue homogenate was found to be significantly elevated ($p<0.05$) in animals treated with Amree Plus® when compared to the normal and diabetic control.

Anti-oxidants are the first line defense and protect the body from free radicals damage. They are also essential for maintaining optimum health and well being.^[39] Human body's antioxidant system is a very sensitive and adaptive, in which GSH is the main constituent. Cell's function and viability is directly proportional to the concentration of reduced form of glutathione (GSH) in the body.^[40] Degradation of glutathione reduces the physiological and anatomical functionality of cell, progressively until cell dies and associated with a number of disease states.^[41] Amree plus® showed the significant ($p<0.05$) enhancement in the GSH level in the tissue homogenates of the normal as well as diabetic rats. So this study showed that the Amree Plus® possesses significant antioxidant activity by reducing free radicals stress and this could help to prevent oxidative damage and promote the healing processes. Similar results of the methanolic extract of the *Argimone maxicana*^[42] and *Rubus ellipticus*^[31] have been reported to increase the levels of anti-oxidants in wounded tissues due to the free radical scavenging activity of the flavonoids present in these plants.

Amree Plus® at higher dose (5400 mg/kg) showed significant results comparable to that of standard drug i.e. Chymoral Forte (36k AU/kg). Although low dose (1200 mg/kg) also showed significant ($p<0.05$) results in all the parameters assessed i.e. percent wound contraction, hydroxy proline, protein content and anti-oxidants. Thus, wound-healing activity of Amree Plus® may be attributed to the

phytoconstituents present in it, which may be either due to their individual or additive effect that hastens the process of wound healing. All these observations reveal that Amree Plus® exhibits significant wound healing activity along with good anti-oxidant profile in normal as well as in diabetic animals. So, the drug may be used for further trials clinically to confirm its wound healing potential.

CONCLUSION

In present work we have found that Amree Plus® showed dose dependent enhancement in % wound contraction as it exhibited significant ($p<0.05$) and consistent wound healing activity at both doses (1200 mg/kg and 5400 mg/kg) when compared to the normal and diabetic control rats. Hydroxy proline, a major component of collagen and protein content was found to be increased significantly ($p<0.05$) in both normal and diabetic rat. Amree Plus® showed direct co-relation between dose and antioxidant activity, when given for 21 days. Both GSH and SOD levels were significantly ($p<0.05$) elevated when compared to control animals. From the above results it can be concluded that the Amree Plus® possesses the significant wound healing activity in diabetes along with antioxidant profile. So this finding should be confirmed further for more precision with large number of animals and small progression of doses and be studied in future for clinical trials, as delayed wound healing is major issue in diabetic patients. There is exigent need of drugs, which can reduce time of delayed healing of wounds in diabetics. So this drug i.e. Amree Plus® may prove a beneficial therapy in diabetic world.

Conflict of interest

We authors declare no conflict of interest in this study/manuscript.

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HOW TO CITE THIS ARTICLE

Thakur S, Dhamija I, Kumar S, Sharma AK, Verma SK. Pharmacological evaluation of wound healing activity of herbal based formulation "Amree Plus®" in diabetic rat. J Phytopharmacol 2017;6(1):1-10.