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Icariin lessened pain perception and ameliorated cutaneous wound healing in rats

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Abstract
Icariin, a flavonoid is a compound extracted from plants of the genus *Epimedium* which are commonly known as Horny goat weed or Yin Yang Huo. The extracts of the plants have been used in many diseases and disorders over the last 2000 years, especially in Chinese traditional medicine. Anti-oxidative, anti-apoptotic, anti-inflammatory, neuron protective, immunoprotective, anti-osteoporotic, aphrodisiac effects are few among the several properties possessed by icariin. There are many reports on icariin as a potential agent in the promotion of damaged bones healing as well as in the prevention and treatment of osteoporosis. Mi and co-workers (2018) injected icariin around cutaneous wound daily for 10 days which enhanced healing in Sprague Dawley rats. Topical application of a drug in ointment base in the treatment of cutaneous wound is relatively easier and less painful when compared to injection. Here, we evaluated the cutaneous wound healing potential of topically applied icariin ointment on day 14 post-wounding in adult Wistar rats.

Keywords: Icariin, wound, healing, antioxidant, post-wounding pain

Introduction
Icariin is a flavonoid compound extracted from plants of the genus *Epimedium* which are commonly known as Horny goat weed or Yin Yang Huo (Singh *et al.*, 2019) [22]. The extracts of the plants have been used in many diseases and disorders over the last 2000 years, especially in Chinese traditional medicine. Recently, investigators have characterised the active principles of the extracts (Ming *et al.*, 2013) [18] and one of them is icariin which produces extensive pharmacological effects in both *in-vitro* and *in-vivo* studies and has shown its potential in the treatment of many diseases/disorders (Ming *et al.*, 2013; Li *et al.*, 2015) [18, 11]. Icariin possesses several properties such as anti-oxidative (Xiong *et al.*, 2014) [23], anti-apoptotic (Deng *et al*., 2017) [6], anti-inflammatory (Zhou *et al*., 2011) [25], neuron protective (Zhang *et al*., 2014) [24], immunoprotective (Li *et al.*, 2011) [12], anti-osteoporotic (Ming *et al.*, 2013) [18], aphrodisiac (Xin *et al*., 2003) [22]. Additionally, icariin has shown potential in promotion of healing of and in the prevention and treatment of osteoporosis (An *et al*., 2016) [2]. Mi and co-workers (2018) [17] reported that injection of icariin around cutaneous wound daily for 10 days enhanced healing in Sprague Dawley rats. Our recent study also revealed enhancement of healing in cutaneous wound in diabetic rats (Singh *et al.*, 2019) [22]. In the present study, we evaluated the cutaneous wound healing potential of icariin in different concentrations in ointment base after topical application on wound twice daily for 14 days in non-diabetic adult Wistar rats.

Material and Methods
Healthy adult male Wistar rats (170 - 200g) were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (U.P.). The animals were housed in polypropylene cages with free access to standard feed and water in divisional animal house, under controlled conditions of temperature (22±2°C), humidity (60-70%), and a 12-hr light/dark cycle, for a week as an acclimatization period. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee, Indian Veterinary Research Institute, Izatnagar.

Wound Model
The animals were anesthetized by an intra-peritoneal injection of ketamine (50 mg/kg) and xylaxine (5 mg/kg) combination. Open excision–type wound of ≈ 2x2 cm^2 (400 mm^2) was created on the back (thoracic region) of the rats to the depth including the panniculus carnosus.

The wound was neither dressed nor covered. Animals were then individually housed in properly disinfected cages.

Drug preparation

Ointment base consisting of soft paraffin (90%), hard paraffin (5%) and lanolin (5%), was used to prepare ointment of icariin (≥94% purity, Sigma-Aldrich, USA).

Grouping of animals and application of ointment

The rats were randomly divided into five groups (*n*=6 in each group). Ointment of icariin was applied topically on the wound area twice daily for 14 days.

- Group 1: Control, 0% icariin
- Group 2: 0.004% icariin
- Group 3: 0.02% icariin
- Group 4: 0.1% icariin
- Group 5: 0.5% icariin

Photographic evaluation and wound contraction measurements

Wounds were photographed and measured on days 0, 3, 7, 11 and 14 post–wounding to assess the quality of wound healing. Surface area was measured by tracing its contour using a transparent sheet.

Assessment of Pain

Post-wounding pain was assessed on days 0, 3, 5 and 7 post-wounding using a scoring system as described in ACF SOP-605.01, Florida International University, USA, with slight modification.

Collection of Tissue

Animals were euthanized on day 14 for collection of healing tissues. One portion of the tissue was preserved in 10% neutral buffer formalin for histopathological evaluation. Second portion was stored at -80ºC for determination of hydroxyproline and glucosamine contents. Finally, third portion of the tissues was homogenized in ice-cold lysis buffer [100 mg tissue in 0.5 ml lysis buffer: 1% Triton X-100, 10 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin and 1 mg/ml leupeptin in phosphate buffer saline (pH 7.4)] and centrifuged at 12,000 rpm for 10 min at 4°C. The aliquots of the supernatant were prepared and stored at -80ºC for antioxidant parameters.

Biochemical and enzymatic measurements

a. **Estimation of hydroxyproline and glucosamine:** Determination of hydroxyproline and glucosamine was done following protocol as reported by Reddy and Enwemeka (1996) [19] and Rondle and Morgan (1955) [20], respectively.
b. **Estimation of healing tissues antioxidants and free radicals:** Protein estimation of the tissue lysate was done using protein estimation Kit (GeNei™, cat. no: 2601800011730) following Lowry's method [13]. The estimation of level of oxidative parameters (both enzymatic and non-enzymatic) was done to determine oxidative damage of the wound. The levels of reduced glutathione (GSH) (Sedlak and Lindsay, 1968), [21] superoxide dismutase activity (SOD) (Madesh and Balasubramanian, 1998) [16] and catalase activity (Aebi, 1984) [1] were determined in healing tissues. The levels of malondialdehyde (MDA) (Buege and Aust, 1978) [3] and nitric oxide (NO) (using Griess Reagent -Fluka, cat. no: 03553) were estimated to determine the extent of lipid peroxidation. The biochemical data were normalized in relation to total protein levels in the supernatant.

Histological analysis of healing tissues

After fixation of skin tissues, the tissue were washed overnight in running tap water, dehydrated in ascending grades of alcohol and cleared in benzene. The 5µm thick sections were cut from paraffin embedded tissue and stained with haematoxylin and eosin stain (H & E) method and Masson's trichrome stain (Lillie, 1940) [14] to confirm gross morphological changes and collagen deposition, respectively, by visualizing under light microscope (OLYMPUS, BX 41, USA) at magnification 10X and 40X. The comparative assessment of the quality of healing wounds was done through a scoring method as per Gal and co-workers (2008) [9] with some modifications.

Statistical analysis

Results were expressed as mean and standard error of mean (mean ± SEM). The level of statistical significance was determined using the GraphPad Prism 6 Software Program (San Diego, CA, USA).

Results

Icariin enhances wound contraction

The measurement of wound contraction (Table 1; Figure 1) on different days of post-wounding indicated positive effect of icariin on wound healing**.** Contraction of wound was non-significantly higher on days 3 and 7 in all the icariin-treated groups, as compared to control. However, measurement on days 11 and 14 showed significantly higher ($p< 0.05$) percentage wound contraction in groups receiving 0.1% and 0.5% respectively, as compared to control.

Table 1: Effect of topical application of different concentrations of icariin on wound contraction in rats.

Treatment	Day 3	Day 7	Day 11	Day 14
Control	6.38 ± 5.23	53.06 ± 2.86	75.47 ± 2.18	85.04 ± 0.74
0.004%	12.36 ± 3.05	59.29 ± 2.12	80.30 ± 1.01	88.74 ± 1.22
0.02%	9.06 ± 4.26	58.22 ± 5.74	82.18 ± 1.37	88.77 ± 1.21
0.1%	11.82 ± 2.85	59.20 ± 4.23	83.55 ± 2.27*	91.28 ± 1.78*
0.5%	15.74 ± 1.15	61.80 ± 2.45	83.73 ± 1.98*	93.35 ± 1.11***

Values are mean ± SEM, (n=6); Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's Post test. *$p< 0.05$ and *** $p< 0.001$, compared to respective control group. SEM= Standard error of the mean, ANOVA= Analysis of variance

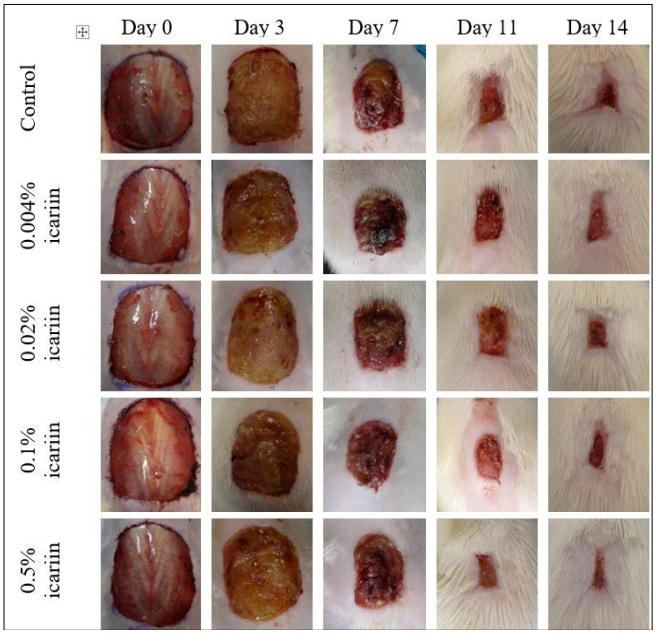

Fig 1: Representative digital photographic evaluation of wound contraction on different days

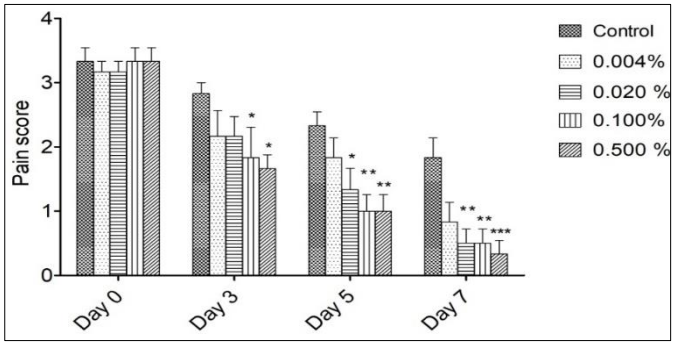

Fig 2: Effect of topical application of different concentrations of icariin on pain perception on different days using pain scores. Data are expressed as mean ± SEM (n=6). Statistical analysis was performed by two-way ANOVA, followed by Bonferroni post test. **p*<0.05, ***p*<0.01, and *** *p*<0.001, compared to respective control group. SEM= Standard error of the mean, ANOVA= Analysis of variance

Icariin reduces pain perception

The application of icariin on cutaneous wounds diminished post-wounding pain perception and was clearly evident from the scoring of pain perception (Figure 2) using the scoring system as described in ACF SOP-605.01, Florida International University, USA, with slight modification. The lessening of pain intensity became significant ($p<0.05$) on day 3 in groups receiving 0.1% and 0.5% icariin, as compared to control. Measurement on days 5 and 7 showed significantly reduced pain in groups receiving 0.02%, 0.1% and 0.5% icariin, as compared to control. The pain became almost imperceptible in some animals in all the icariin-treated groups, except 0.004% icariin group, on day 7 post-wounding, as compared to control.

Icariin reduces tissue damages and promotes healing

The levels of antioxidants and free radicals determined in the healing tissues collected on day 14 post-wounding are given in Table 2. Different concentrations of icariin caused increased level of antioxidants and decreased levels of oxidants. The levels of GSH (nM/mg protein) and catalase activity (IU/mg protein) were significantly higher in all the icariin-treated groups, except in group receiving the lowest concentration (0.004%), as compared to control. SOD (IU/mg protein) level was also increased in all the icariin-treated groups, where it was significantly more ($p<0.001$) in groups-IV (0.1%) and V (0.5%), as compared to control. The level of MDA (nM/mg protein) was non-significantly reduced in the first three lower icariin-concentration groups, as compared to control. However, the group treated with the highest icariin concentration (0.5%) showed significantly reduced level of MDA, as compared to control. NO (nM/mg protein) levels were significantly reduced in all the icariin-treated groups at different level of significance, as compared to control.

Table 2: Status of antioxidants and free radicals in control and icariin-treated groups.

Treatment	Antioxidants			Free radicals	
	GSH nM/mg protein	SOD U/mg protein	CAT nmol/min/mg protein	MDA nM/mg protein	NO µM/mg protein
Control	15.92±2.142	11.58±0.93	179.66±2.90	0.86±0.05	45.12 ±1.31
0.004%	34.47±6.89	14.37±1.16	197.55±4.16	0.65±0.03	23.39 ±5.56**
0.02%	39.90±3.20*	16.17±0.44*	274.34±5.50***	0.62±0.02	15.98 ±1.28***
0.1%	52.70±2.18**	16.28±0.53*	322.77±2.84***	0.45±0.13	13.96±2.04***
0.5%	56.06±6.38***	18.38±0.58***	396.55±3.07***	0.23±0.05**	13.65 ±1.87***

Values are mean ± SEM, (n=6); Statistical analysis was performed by one-way ANOVA, followed by Dunnet's multiple comparison test. **p*<0.05, ***p*<0.01, and *** *p*<0.001, compared to respective control group. SEM= Standard error of the mean, ANOVA= Analysis of variance, GSH=Reduced glutathione, SOD= Superoxide dismutase, CAT= Catalase, MDA= Malondialdehyde, NO= Nitric oxide

Icariin increases levels of glucosamine and hydroxyproline

Both glucosamine and hydroxyproline levels were significantly higher in the icariin-treated groups, except in the lowest icariin concentration, as compared to control (Figure 3).

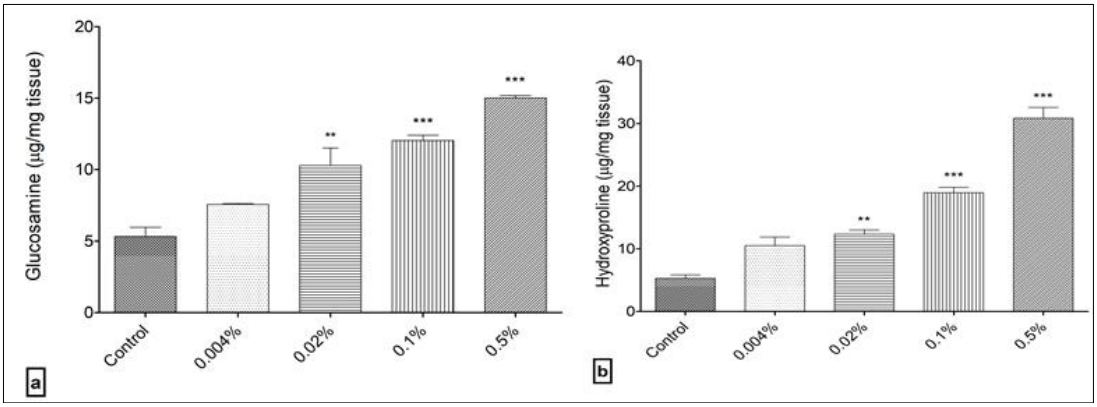

Fig 3: Status of Glucosamine (a) and Hydroxyproline (b) in healing tissues of control and icariin-treated rats on day 14 post-wounding. Data are expressed as mean ± SEM (n=6). Statistical analysis was performed by one-way ANOVA, followed by Dunnet's multiple comparison test. ***p*<0.01 and ****p*<0.001, compared to respective control group. SEM= Standard error of the mean, ANOVA= Analysis of variance.

Histological Study

Histological analysis of the wound tissues was carried out using hematoxylin and eosin stain (Figure 4a_1 and 4a_2) as well as Masson's trichrome stain (Figure 4b). Semi-quantitative analysis was done as per the method given by Gal and co-workers (2008) [9] to evaluate histological processes and structures which include-re-epithelisation, polymorphonuclear leucocytes, fibroblasts, new vessels and new collagen. The sections were evaluated in the scale as 0, 1, 2, 3 and 4 (Table 3) by three independent observers. The mean value was used for statistical comparison. The granulation tissues from rats in control group contained high number of inflammatory cells, which were comparatively reduced after topical application of different concentrations of icariin in groups (II-V). The proliferation and migration of fibroblast were also increased in all icariin-treated groups, as compared to control. The angiogenesis in the granulation tissues was more pronounced in all icariin treated groups, compared to control. Masson's trichrome staining intensities distinctly revealed significantly enhanced formation and deposition of blue coloured well organised collagen fibers in all the icariin-treated groups, except in 0.004% icariin, as compared to control (Figure 5). The epithelial layers were also thicker in the icariin-treated groups, as compared to control group.

Table 3: Semi-quantitative evaluation of histological sections using scale given by Gal *et al.*, 2008 [9]

Parameters	Control	0.004% icariin	0.02% icariin	0.1% icariin	0.5% icariin
Epithelisation	3.00±0.25	3.00±0.25	3.33±0.21	3.50±0.22	3.66±0.21
PMNL	0.66±0.21	0.50±0.22	0.33±0.21	0.16±0.16	0.16±0.16
Fibroblasts	1.00±0.36	1.50±0.34	1.50±0.34	3.00±0.36***	3.00±0.51***
New vessels	2.33±0.42	2.50±0.34	3.0±0.51	3.00±0.63	3.00±0.36
Collagen	2.16±0.47	2.33±0.42	3.00±0.51	3.33±0.33*	3.5±0.34*

Values are mean ± SEM; Statistical analysis was performed by one-way ANOVA, followed by Dunnet's multiple comparison test. **p*<0.05 and ****p*<0.001, compared to respective control group. SEM= Standard error of the mean, ANOVA= Analysis of variance, PMNL= Polymorphonuclear leukocytes

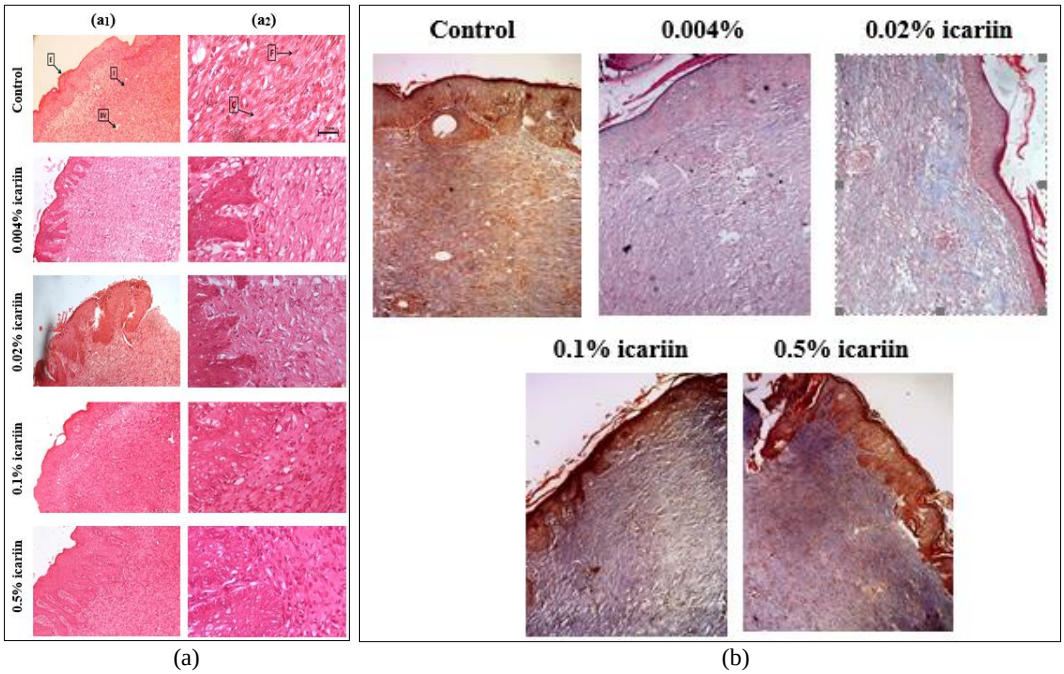

Fig 4: Digital photographic images of H & E stained sections: 10x (a_1) and 40x (a_2), and Masson's trichrome stained sections (10x) (b), (E=epithelisation; I=polymormhonuclear leucocytes; BV= blood vessels; C=collagen and F=fibroblast)

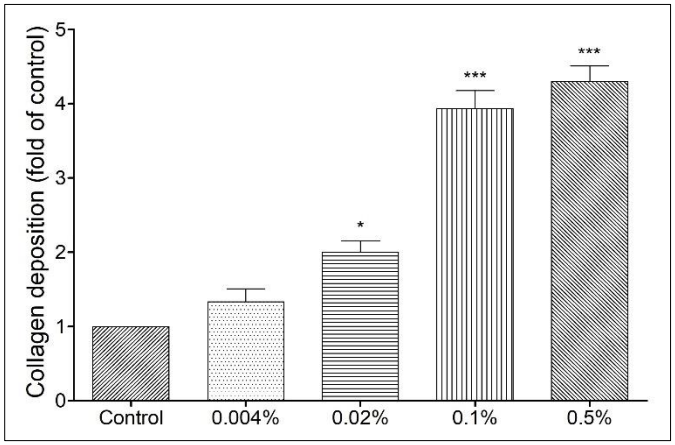

Fig 5: Relative collagen deposition in healing tissues of control and icariin-treated rats on day 14 post-wounding. Data are expressed as mean ± SEM (n=6). Statistical analysis was performed by one-way ANOVA, followed by Dunnet's multiple comparison test. *p<0.05 and ***p<0.001, compared to respective control group. SEM= Standard error of the mean, ANOVA= Analysis of variance.

Discussion

Icariin is a flavonoid which has been used in various human ailments since time immemorial because of its multiple medicinal values. The use of this compound as an aphrodisiac since ancient time as well as in the treatment of bone diseases (osteoporosis) and cancer in recent years is well known. In our present study, we investigated the wound healing effects of icariin when topically applied daily (b.i.d.) for 14 days in Wistar rats. The treatment resulted in enhancement of wound contraction. This finding was also supported by the upregulation of antioxidants (GSH, CAT and SOD) with the concomitant reduction of the level of oxidants (MDA and NO) in all the icariin-treated groups. The crucial role of a delicate balance between antioxidants and oxidants in wound healing is well known and this has also been reported by many investigators (Kurahashi *et al.*, 2015; Fitzmaurice *et al.*, 2011) [10, 8].

As expected, histological analysis also revealed increased angiogenesis, collagen deposition, and decreased infiltration of PMNL in granulation tissues of all the icariin-treated groups, as compared to control group. Similar findings have also been reported by El-Ferjani and co-workers (2016) [7], in which wound treated with topical application of new Schiff base derived Co (II) complex in rats showed increased collagen deposition, angiogenesis and fewer inflammatory cells in healing tissue.

The epidermal thickness was significantly more in icariin-treated groups, as compared to control. This was consistent with the previous study conducted by Mi and co-workers (2018) [17]. Our experiment also surprisingly indicated post-wounding pain perception lessening effect of icariin. This could have possibly resulted due to faster healing of wound in the icariin-treated groups. However, neuronal protective/ regenerative and anti-inflammatory effects of icariin might also be contributing in reducing pain perception. The various mechanisms for neuroprotective nature of icariin have been reported by many investigators (Chung *et al*., 2008; Li *et al*., 2010) [5, 13]. Nerve growth factor releasing effect of topically applied icariin is one responsible for promoting peripheral nerve regeneration in spinal injury (Chen *et al*., 2015) [4].

Conclusion

Our investigations revealed modulating effects of icariin on multiple cells and molecules. The modulation of the level of antioxidants and oxidants, and subsequent subsiding of inflammation and other cell damaging effects have found to play crucial role, upto certain extend, in the enhancement of wound healing. Nevertheless, its effects on the proliferation and migration of cells, and deposition of collagen as well as reduction of pain perception should not be neglected when exploring the detailed molecular mechanisms on wound healing in normal as well as in diseased models.

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Conflicts of interest

There are no conflicts of interests.

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