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Article

Wound healing activity in rabbits and antimicrobial activity of *Hibiscus hirtus* ethanolic extract

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Generally, Plants have immense potential in the wounds management and treatment.In Chinese herbology, *Hibiscus* plant is a potent herb and have a good medicinal values but not scientifically approached. The present study aims to investigate the wound healing and antimicrobial activity of ethanolic extract of *Hibiscus hirtus* Linn.(*HH*). Wound healing activity was carried out by excision, incision and burn wound models. Antimicrobial activity was determined by cup plate method. Healing rate was assessed from the rate of wound contraction, epithelialization rate, tensile strength, hexosamine and hydroxyproline content. From the obtained results, it was indicated that the wound contraction and increased tensile strength of *Hibiscus hirtus* extract exhibits potent wound healing capacity. Hexosamine and hydroxyproline expression were also correlative with the pattern of healing observed. Histological observation indicates that the wounds treated with *Hibiscus hirtus* extract and povidone iodine have reduced scar formation and enhances fibroblast proliferation, angiogenesis, keratinization and epithelialization. The *Hibiscus hirtus* extract has excellent antimicrobial activity against the various organisms. Wound healing activity of our ethanolic extract of *Hibiscus hirtus* has shown the good effect which has proved by different physical, histological, biochemical parameters. Significant antimicrobial activity shown may be due to major active constituents present in plant.

Keywords: *Hibiscus hirtus*/wound healing activity/antimicrobial activity. Medicinal plants.

INTRODUCTION

Plants have immense potential in the wounds management and treatment. To treat various wound injuries and skin disorders, many plants are used as traditional medicines (Swamy *et al.,* 2007; Harish *et al.,* 2008; Sharath *et al.,* 2010; Lingaraju *et al.,* 2012). Wounds are physical injuries that lead to an opening or breaking of the skin. Wound healing processes consist of integrated cellular and biochemical cascades leading to reestablishment of structural and functional integrity of the damaged tissue (Boateng *et al.*, 2008) The injured tissues repair occurs as a sequence of events, which consists of inflammation, proliferation, and migration of different cell types (Sidhu *et al.,* 1999). The inflammatory stage begins after an injury, initially with vasoconstriction that causes homeostasis and releases various mediators of inflammation. The proliferative phase mainly consists of a proliferation of granulation tissue formed by fibroblast and the angiogenesis process. Reformulations and improvement in the components of the collagen fiber that increases the tensile strength are characteristics of remodeling stage (Varoglu *et al.,* 2010). Poor perfusion or oxygenation, Repeated trauma, and excessive inflammation are the factors that are responsible for the causation and perpetuation of the chronicity of wounds (Harding, Moore, Phillips, 2005). Various growth factors such as transforming growth factor beta (TGF-β), platelet activation factor (PAF), epidermal growth factor (EGF), and platelet-derived growth factors (PDGF) seem to be necessary for the initiation and promotion of wound healing (Menke *et al.,*2007)

The plant constituents are having better compatibility with the human body as they are a part of the physiological function of living flora (Kamboj, 2000). Many Scientists are looking towards the Indian traditional system of medicineThe present treatment for the wounds includes

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the application of silver products, steroids, advanced skin substitutes and dressings, growth factor, negative pressure wound devices, and hyperbaric oxygen (Murphy, Evans, 2012). In the management and treatment of wounds, plants have immense potential. Several plants have been experimentally used to treat skin disorders and wound injuries as traditional medicines (Swamy *et al.,* 2007; Harish *et al.,* 2008; Sharath *et al.,* 2010).

Hibiscus genus is the flowering plants in the mallow family, Malvaceae. The genus contains several hundred species that are native to warm-temperate, subtropical, tropical regions throughout the world and is quite large. *Hibiscus* plant has a number of medical uses in Chinese Herbology. Their research indicates some potential in cosmetic skin care; for example, an extract from the flowers of *Hibiscus plant* has been shown to function as an anti-solar agent by absorbing ultraviolet radiation (Sidram, Lokapure, Kalyane, 2011).

In the Indian traditional system of medicine, Ayurveda, *Hibiscus*, especially white *Hibiscus* and red *Hibiscus* are considered to have medicinal properties. The roots are used to make various concoctions believed to cure ailments such as cough, hair loss or hair graying. The leaves and flowers are ground into a fine paste with a little water, and the resulting lathery paste is used as a shampoo plus conditioner. A previous animal study demonstrated the effects of the *Hibiscus plant extract* on atherosclerosis in rabbits. Notably, a reduction in triglyceride, cholesterol, and low density lipoprotein was observed in rabbits consuming a high cholesterol diet (HCD) in addition to *Hibiscus plant* extract compared to rabbits only fed HCD, suggesting a beneficial effect (Chen, Hsu, Wang, 2003). Furthermore, the *Hibiscus* seed is abundant in phytosterol and tocopherol, plant forms of cholesterol that has antioxidant and LDL cholesterol lowering effects (Mohamed *et al.,* 2007). Based on the literature review,so far no work has been carried out in *Hibiscus hirtus* Linn. *(HH).* Antimicrobial and wound healing activities have been reported in some species of the genus *Hibiscus*.

Therefore the present investigation carried out an in-depth study regard the *H. hirtus* ethanolic extract wound healing activities using incision, excision, and burn wound models and evaluated its antimicrobial potential against pathogenic microorganism.

MATERIAL AND METHODS

Collection of plant

The whole plant of *HH* was collected near the Pithapuram, Andhrapradesh, India which is located at India country in the Castles place category with the gps coordinates of 17° 6' 44.7372'' N and 82° 15' 10.4364'' E during November 2014. The plant was authenticated by Dr.S.B.Padal, Associate Professor, Botany Department, Andhra University and given a voucher specimen number as 22205.

Preparation of ethanolic extract of HH

The freshly collected whole plants of *HH* were cleared of dirt and dried under shade for about 20 days and the grindered into powder using a mechanical grinder. The powder was extracted with 95% ethanol (was used for extraction as many polar and non polar compounds can be extracted from the ethanol) for 3 days, followed by hot percolation for 3 hrs. Then it was filtered and distilled at 80 °C. Then it was transferred into the previously weighed empty china dish and evaporated to get a ethanolic extract and kept in anhydrous calcium chloride containing desiccator. The percentage yield of the extract was calculated (Khandelwal, 2008).

Preparation of HH ointment

Based on literature review on other *Hibiscus* species (Bhaskar, Nithya, 2012) the extract was prepared with two concentrations of extract containing ointment (10% and 20% w/w). The 10% ointment of *HH* consists of plant extract 2.5 g and 22.5 g of ointment base(white bees wax 1.5 gm and white soft paraffin 21.0 g). The 20% ointment of *HH* consists of plant extract 5 g, and 20gm.of ointment base (white bees wax 1.5 g and white soft paraffin 21.0 g)

Preliminary phytochemical testing

Preliminary phytochemical screening of *HH* extract was done to test the presence of the active chemical constituents such as alkaloids, flavonoids, tannins, phenolic compounds, saponins, fixed oils and fats (Khandelwal, 2004).

Quantitative phytochemical testing

Etimation of phenolic contents

The phenolic contents of ethanolic extract of *HH* were determined by using the method Folin-ciocalteu (Singleton, Orthofer, Lamuela-Raventós, 1999). An extract of 0.5 mL was mixed with 3 mL Folin-ciocalteu reagent (1:10 v/v). Allow it for 5 min, and then add 4 mL of (20% w/v) of sodium carbonate solution. The tubes were kept aside for 15minutes at 30 °C temperature for colour development. Read at 765 nm by spectrophotometer.

Phenolic content were estimated from the calibration curve using standard gallic acid in methanol and the results were expressed as gallic acid equivalent mg/100 mg dry weight of extract.

Estimation of total flavanoids

Total flavonoid contents of *HH* ethanolic extract was determined by aluminium chloride method (Chang *et al.,* 2002). To 0.6 mL of the extract, add 1.8 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M sodium acetate, 3 mL of distilled water and left at 30 °C temperature. Note the absorbance after 30 min at 415 nm. Total flavanoids were estimated from the calibration curve using standard quercetin in methanol and the results were expressed as quercetin equivalent mg/100 mg dry weight of extract.

Estimation of total anthocyanin

Total anthocyanin content (TAC) of freeze-dried extract was determined using the method described by (Česonienė *et al.,* 2012). 10 mg of freeze-dried extract was mixed in 5 mL of methanol acidified with trifluoroacetic acid 0.1% (v/v). Aliquots of the extracts were taken in a10 mL glass tube and adjust to a volume of 3 mL with methanol acidified with trifluoroacetic acid (TFA) and the absorbance was measured at 530 nm using a UV/Vis spectrophotometer against the blank sample containing the mixture methanol/TFA 0.1% without the sample extract, TAC was estimated as cyanidin 3-*O*-glucoside at 530 nm using a molar extinction coefficient of 26,900 L/mol/cm) and molar mass (449 g/mol) and was expressed as mg cyanidin-3-glucoside (mg Cya3G)/g of freeze-dried extract (g FDE).

Experimental animals

Rabbits of either sex weighing about 1.8-2.5 kg were used for the study. Three animals are used in each group in each model and totally 36 animals were used. All the animals are properly caged and maintained under standard pellet diet and water ad libitum, placed in a properly air conditioned room with 12hrs light and dark cycles. The animal experiments were performed based on the Institutional Ethics Committee (IEC) approval and guidelines REG. No. 1269/a/10/CPCSEA.

Wound healing activity

Excision, burn and incision wound healing models were used to evaluate the wound healing activity of *HH* Animals were divided into four groups of 3 animals (n = 3) (Aderounmua *et al.,* 2013). Povidone iodine ointment (5%w/w in oleaginous ointment base (white bees wax 1.5 gm and white soft paraffin 21.0 g)). is used as standard treatment as it is a well reported antimicrobial agent and is used to prevent secondary wound infections (Khan *et al.,* 2015).

Incision wound model

One parallel 6cm paravertebral incisions were made through the full thickness of the skin, 1cm lateral to the mid line of the vertebral column after giving anesthesia (Agarwal *et al.,* 2009). The wound was closed with interrupted sutures 1cm apart with the help of black silk thread and a curved needle (no.11). The sutures removed on the 7th post wounding day. Wound breaking strength (WBS) was measured on the post wounding day in anesthetized rabbits. Standard weights were put slowly and steadily into the S-shaped hook. A gradual increase in weight was transmitted to the wound side hook apart the wound edges. As and when the wound was just opened up, the weight was stopped and noted. Three readings for a given incision wound were recorded and the entire procedure was repeated again. The average reading of the group was taken as an individual value of breaking strength. Average value indicates the breaking strength for respective group.

Excision wound model

Rabbits were anesthetized with lignocaine and a wound was made in an area about 500 mm^2. Full thickness of the marked skin was then cut carefully. Wounds were traced on 1 mm^2 graph paper, initially on the first day of wounding and up to at a gap period of 4 days till 12^{th} day. Then on alternate days healing was complete. Changes in the area of wound were measured periodically and the rate of contraction of the wound was calculated as given in the formula below

$$Percentage\ Wound\ Contraction = 100 - \left[\frac{Final\ diameter\ (cm) \times 100}{initial\ diameter\ (cm)}\right]$$

Significance in wound healing of the test treated groups is derived by comparing the wound area healed on respective days with the negative control groups. The period of *epithelialization* was recorded (Nakae, Inaba, 2000)

Burn wound model

Partial thickness of burn wounds were inflicted on overnight in starved animals under lignocaine anesthesia by pouring the hot molten wax (2 g) at 80 °C. The wax was poured on the animal shaven area through a cylinder of 300 mm^2 circular opening. The wax gets to remain on the

skin till it gets solidified. Immediately after the injury and on subsequent days, the drug or base was applied topically as mentioned (Rashed, Afifi, Disi, 2003).

$$Percentage\ Wound\ helaing = 100 - \left[\frac{Final\ diameter\ (cm) \times 100}{initial\ diameter\ (cm)}\right]$$

Biophysical parameters

In the excision wound model the rate of wound contraction was determined as a percentage reduction of the wound size and the surface area was measured on the 0th, 3rd, 6th, 9th, 12th, 15th, 18th and 21st post-wounding days by measuring the wound on a transparent graph sheet. The period of epithelialization was also noted (Ghosh *et al.,* 2012). The degree of wound healing is represented by tensile strength of the wound. It describes how much the repaired tissue resists to breaking under tension and may indicate the quality of repaired tissue. The incision wound tissues tensile strength was measured on the 10th day by Lee's method after the sutures removal on the 8th post-wound day (Lee, 1968).

Biochemical parameters

A piece of skin from the healed wound area was collected on day 4, 8 and 16 of the post surgery of excision, and analyzed for hydroxyproline content, which is a basic constituent of collagen. Tissues were dried in the hot air oven to constant weight at 60–70 °C and were hydrolyzed in 6 N HCl at 130 °C for 4 h in a sealed tube. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine T oxidation for 20 min, by addition of 0.4 M per chloric acid the reaction was terminated and color was developed with the help of ehrlich reagent at 60 °C (Woessner, 1961) and measured at 557 nm using UV/Vis spectrophotometer following a method mentioned by Johansen with minor modifications (Johansen, Marshall, Neuberger, 1960).

Histopathology

Deep granulation tissues from the implanted tube and the cross-sectional full-thickness skin specimens were collected on the 10th day for the histopathological alterations. The samples were kept in 10% buffered formalin, processed, blocked with paraffin, then sectioned into 5µm sections, and stained with hematoxylin and eosin.

Microbial cultures

These were procured from Microbes Speciality Lab Danavaipeta, Rajahmundry, East Godavari District 533103, Andhra Pradesh, India are aseptically maintained in our laboratory. The gram positive bacteria are *Staphylococcus aureus (ATCCBAA 1026), Bacillus subtilis (ATCC 11774) and Staphylcoccuswerneri (ATCC 27836). The* gram negative bacteria used in the study are *Eschereria coli (ATCC 10536), Klebisella pneumonia (ATCC 33495), Pseudomonas aurgenosa (ATCC 10662), Pseudomonas putida (ATCC 700007)* and *Proteus mirabilis (ATCC 14153).* The fungal strain used is *Candida albicans (ATCC 10231).*

Antimicrobial activity

The antimicrobial screening is based on measuring the diameters of the zones of inhibition around the cylindrical cups incubated with different concentrations of ethanolic extracts of the *HH* For this sabouraud dextrose agar plate (SDA) seeded with microbial cultures were used. A sterile borer was used to prepare cups of 6 mm diameter in the agar medium spread with the micro-organisms and 0.1 mL of inoculum was spread on the agar plate by spread plate technique. 50 µL ethanolic extracts concentration of 50,100,150 mg/mL dissolved in DMSO were filled in the wells. For antibacterial and antifungal activity, gentamycin (25 µg/mL) and flucanozole (25 µg/mL) were used as standard drugs. All the plates were kept in a refrigerator at 2 to 8 °C for a period of 2 h for effective diffusion of test compounds and standards. Later, they were incubated at 37 °C for 24 h. The assay was carried out in triplicate. The diameter of the zone of inhibition was measured and recorded (Indian Pharmacopoeia, 1996).

MIC was determined by the microbroth dilution method. In each well of microtiter plate, specifically 0.1 mL of standardized inoculums of bacteria (CFU/mL) was added which was incubated aerobically at 37 °C for bacterial growth for 18–24 h. MIC means the lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with that of negative control (Sharma *et al.,* 2007).

Statistical analysis

Statistical comparison was carried out using either unpaired t-test or one-way analysis of variance (ANOVA) followed by post tests like Dunnett's test for multiple comparisons.

RESULTS

Preliminary and quantitative phytochemical screening

The extraction yield of ethanol extract was found to be 9.6% w/w. The extract was dark green in colour. The

results of preliminary phytochemical analysis of ethanolic extract of *HH* indicated the positive result for all classes of secondary metabolites like alkaloids, anthocyanins, flavonoids, saponins, tannins, carbohydrates and proteins.

Then *HH* extract was investigated for the total phenolic compounds, total flavonoid content and anthracyanin content using UV. The results of phenolic content by UV visible spectroscopy was expressed by mean±standard deviation. The quantitative phytochemical determination of ethanolic extract of *HH* contains phenolics (7.40 ± 0.50 mg/g) , flavanoids(3.50 ± 0.85 mg/g), anthocyanins (18.53 ± 1.10 mg/g)

Wound healing activity

The wound healing contracting ability of ethanolic extract of *HH* of different concentrations by using oleaginous ointment base on excision wound, incision wound and burn wound models was significantly greater than that of the negative control group. The extract ointment treated groups showed significant wound healing from 3rd day onwards which was comparable to that of standard drug i.e. povidone iodine ointment treated group in all three wound models.

Incision wound model

The 20% extract of ointment treated groups showed significant tensile strength on 10th post wounding day was comparable with that of standard drug i.e. povidone iodine ointment treated group. The maximum tensile strength was observed in (17.69±0.20) g which almost similar to that of povidone iodine ointment treated group (20.43±0.05) g. The 10% extract group showed maximum tensile strength was observed in (15.45±0.15) g as shown in the Table I and Figure 1.

Excision wound model

The wound closure time was less, as the percentage of wound contraction was rapid with high dose of *HH*

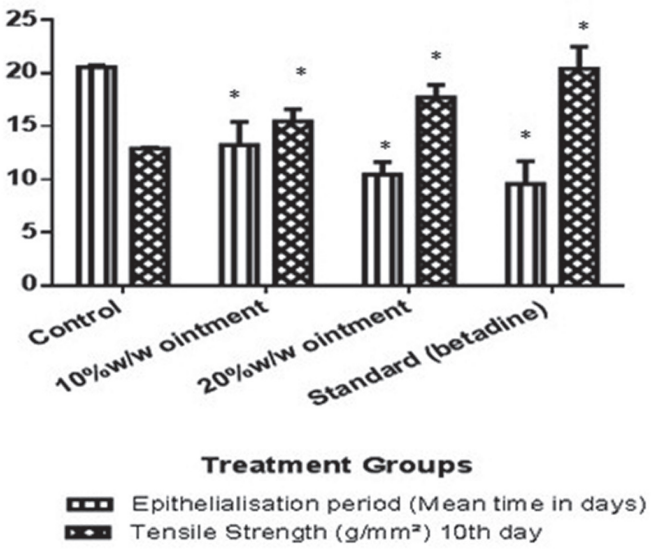

FIGURE 1 - Measurement of epithelialisation and tensile strength in incision wound model.

and 20%w/w extract group exhibited 100% contraction in (16.24±0.21) days during excision wound healing which is almost similar to that of povidone iodine ointment treated group (15.91±0.12) days. The 10%w/w extract group of animals showed significant wound contraction from 6th day onwards and achieved 100% wound closure in (20.8±0.23) days as shown in Table II and Figure 2. The results of the present study revealed that both concentrations (20% extract ointment and 10% extract ointment) of an ethanolic extract of *HH* have significant wound healing activity in excision model.

Determination of hydroxyproline and hexosamine content

The hexosamine and hydroxyproline content of granulation tissue of 4, 8 and 16 post surgery days are indicated in Table III. A significant increase in the hydroxyproline content was observed in *HH* treated

TABLE I - Effect of topical application of ethanolic extract of Hibiscus hirtus on healing of incision wound model in rabbits

Groups	Epithelialisation period (Mean time in days)	Tensile Strength (g/mm²) 10th day
Negative control	20.58±0.16	12.89±0.11
Ethanolic extract of *H. hirtus*(10%w/w ointment)	13.21±2.22*	15.45±1.15*
Ethanolic extract of *H. hirtus*(20%w/w ointment)	10.45±1.19*	17.69±1.20*
Standard (Povidone iodine ointment)	9.56±2.15*	20.43±2.05*

Values are expressed as mean ± SEM; n=3 animals in each group; * $p < 0.05$. Statistically significant difference in comparison with negative control group.

TABLE II - Effect of topical application of ethanolic extract of *Hibiscus hirtus* on healing of excision wound model

Group	Post wounding days								Period of Epithelialisation
	0-day	3rd day	6th day	9th day	12th day	15th day	18th day	21st day	
Negative control	2.56±0.09	2.39±0.03	2.12±0.08	1.52±0.03	1.21±0.04	0.91±0.03	0.79±0.03	0.54±0.02	24.68±0.16
Ethanolic extract of *H. hirtus*(10%w/w ointment)	2.54±0.08	2.32±0.09	2.01±0.07	1.31±0.09	0.90±0.07	0.61±0.08	0.32±0.09*		20.8±0.23*
Ethanolic extract of *Hibiscus hirtus*(20%w/w ointment)	2.55±0.11	2.24±0.10	1.76±0.11	1.09±0.13	0.33±0.09	0.12±0.03*			16.24±0.21*
Standard (Povidone iodine ointment)	2.59±0.01	2.12±0.04	1.52±0.03	0.92±0.06	0.21±0.05	0.09±0.02*			15.91±0.12*

Values are expressed as mean±SEM;n=3 animals in each group; * $p < 0.05$. Statistically significant difference in comparison with negative control group.

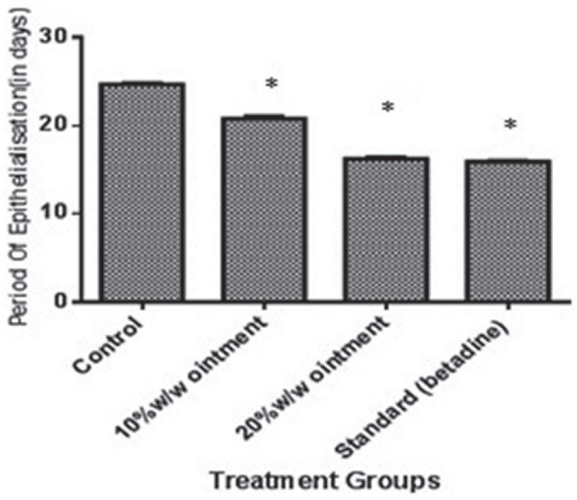

FIGURE 2 - Epithelialization Periods in different treatment groups in excision wound models.

groups than negative control groups. Throughout the healing course, hexosamine and hydroxyproline content were found to be increased in all treated groups compared to the negative control group, which are a very important constituent of the extracellular matrix for the healing process. These components are good markers for wound healing activity. The hydroxyproline and hexosamine content of granulation tissue of 4, 8 and 16 post surgery days are given in Table III. A significant increase in the hydroxyproline content was observed in *HH* treated groups than that of negative control groups. Throughout the course of healing, hydroxyproline and hexosamine content were found to be more in all treated groups than negative control group, which are important constituents of the extracellular matrix for healing. These compounds are good markers for wound healing.

Burn wound model

The percentage of wound contraction was greater with 20% dose of *HH* and 100% wound contraction was observed in (14.4±0.89) days, which almost similar to that of povidone iodine ointment treated group (13.67±0.38) days. The low dose extract group of animals showed significant wound contraction from 6th day onwards and achieved 100% wound closure in (18.7 ±0.22) days as shown in Table IV and Figure 3.

Histopathalogical studies

The granulation tissue provides further evidence

TABLE III - Hexosamine and hydroxyproline content of granulation on different days of healing

Treatments	Hexosamine (mg/100 mg of tissue)			Hydroxyproline (mg/g tissue)		
	4th day	8th day	16th day	4th day	8th day	16th day
Negative control	0.24±0.30	0.44±0.05	0.65±0.08	23.6±3.11	31.5±1.08	41.8±1.21
Ethanolic extract of *H. hirtus* (10%w/w ointment)	0.31±0.03	0.56±0.03	0.66±0.02*	29.6±1.09	44.8±2.15	62.8±1.37*
Ethanolic extract of *H. hirtus* (20%w/w ointment)	0.42±0.03	0.75±0.02	0.81±0.01*	39.3±1.19	53.8±2.15	74.9±2.27*
Standard (Povidone iodine ointment)	0.45±0.07	0.83±0.04	0.87±0.04*	43.4±1.09	58.9±4.03	81.8±2.17*

Values are expressed as mean±SEM;n=3 animals in each group;* $p < 0.05$. Statistically significant difference in comparison with negative control group.

TABLE IV - Effect of topical application of ethanolic extract of *Hibiscus hirtus* by using oleaginous ointment base for healing of burn wound model

Group	Post wounding days								Period of Epithelialisation
	0-day	3rdday	6thday	9thday	12thday	15thday	18thday	21stday	
Negative control	2.65±0.01	2.57±0.01	2.24±0.02	1.95±0.01	1.54±0.02	0.89±0.02	0.75±0.03	0.54±0.01	23.69±0.12
Ethanolic extract of *H. hirtus* (10%w/w ointment)	2.64±0.90	2.55±0.08	2.12±0.08	1.84±0.09	1.33±0.10	0.65±0.12*			18.7±0.22*
Ethanolic extract of *H. hirtus* (20%w/w ointment)	2.63±0.10	2.46±0.10	1.93±0.09	1.24±0.09	0.25±0.01*				14.4±0.89*
Standard (povidone iodine)	2.67±0.01	2.41±0.04	1.74±0.02	0.95±0.02	0.11±0.01*				13.67±0.38*

Values are expressed as mean±SEM; n=3 animals in each group; * $p < 0.05$. Statistically significant difference in comparison with negative control group.

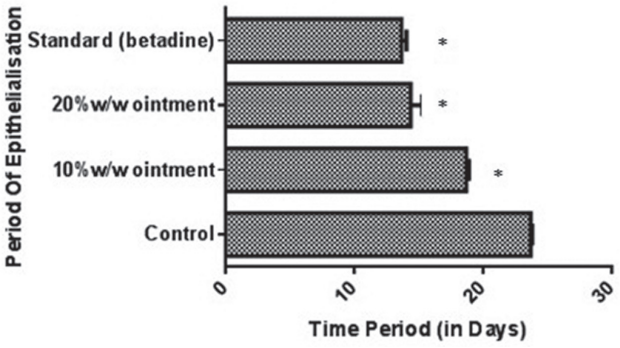

FIGURE 3 - Epithelialization Periods in different treatment groups in Burn wound models.

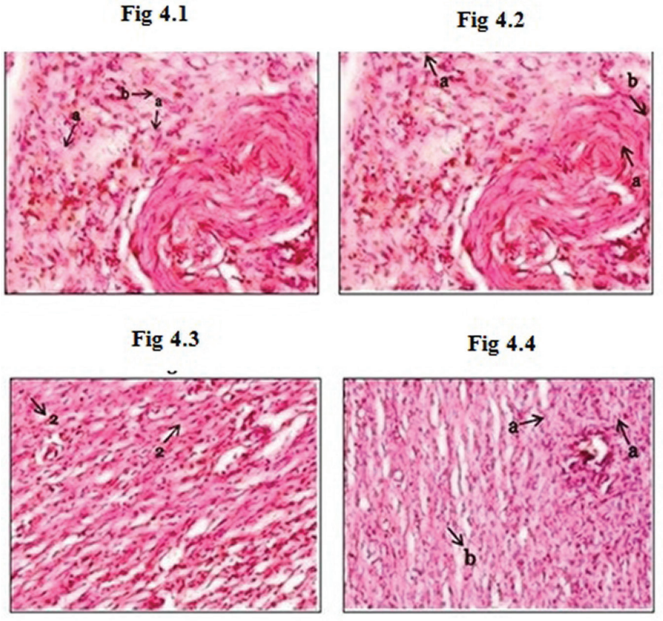

FIGURE 4 - Histological examination.

for the wound healing efficacy of the *HH* extracts. The granulation tissue section of the negative control animals showed lower epithelialization and collagen formation with a greater macrophage aggregation indicating the incomplete wound healing (Figure 4.1). The sections of granulation tissue obtained from the *HH* (10%w/w ointment) treated animals showed a significant rise in collagen deposition, a few macrophages and more fibroblasts (Figure 4.2). The high deposition of collagen and a significant decrease in infiltration of macrophage were observed in the wound tissue section treated with *HH* (20%w/w ointment) (Figure 4.3). The animals treated with povidone iodine ointment showed increased collagenation and depletion in the accumulation of macrophages at the site of the wound (Figure 4.4).

Antimicrobial activity

The whole plant extract of *HH* has shown inhibition effects on the growth of all the organisms tested, but their efficiency in inhibitory was varied between the organisms. *HH* has shown inhibition diameter from 12.4 to 18.3 mm. *Escherichia coli* (18.6±0.33mm) was most sensitive to *HH* followed by *Proteus mirabilis* (18.3±0.33 mm), *Bacillus subtilis* (18.0±0.57 mm), *Pseudomonas putida* (17.6±0.57 mm), *Klebesiella pneumonia* (17.0±0.57 mm), *Staphylococcus aureus* (16.6±0.33 mm), *Staphylococcus werneri* (16.3±0.33 mm), *Pseudomonas aerugenosa* (16.3±0.67 mm) and *Candida albicans* (14.2±0.12 mm) . The antimicrobial activity results are indicated in Table V and Figure 5. The ethanolic extract of *HH* has shown minimum inhibitory concentration at 125mg/mL against gram negative organism *Escherichia coli.*

DISCUSSION

In terms of morbidity and mortality, wound represents as a major health problem. In the present investigation, three different models were used to assess the wound healing effect of *HH* extracts on various phases of wound healing. So far no data have been reported on this plant product for wound healing property. The

TABLE V - Zone of inhibition of ethanolic extract of *Hibiscus hirtus*

Type of bacteria	Name of microorganism	Zone of Inhibition (mm)		
Antibacterial activity		***Hibiscus hirtus*** **(100 mg/mL)**	***Hibiscus hirtus*** **(200 mg/mL)**	**Gentamycin (25 µg/mL)**
Gram positive bacteria	*Staphylococcus aureus*	15.0±0.88	17.0±0.35*	19.0±0.58
	Bacillus subtilis	15.3±0.58	17.0±0.57*	21.0±0.33
	Staphylococcus Wernerı	14.4±0.58	16.3±0.38*	20.0±0.45
Gram negative bacteria	*Escherichia colı*	17.0±0.33	19.0±0.41*	21.3±0.37
	Klebesiella pneumonia	14.5±0.45	17.0±0.57*	18.0±0.53
	Pseudomonas aerugenosa	14.0±0.67	16.3±0.67*	19.0±0.57
	Pseudomonas putida	14.3±0.36	18.0±0.54*	19.0±0.58
	Proteus mirabilis	15.3±0.33	18.0±0.39*	20.3±0.45
Antifungal activity				**Flucanozole (25 µg/mL)**
Fungal strain	*Candida albicans*	12.4±0.15	14.2±0.12*	17.4±0.21

Values are expressed as mean ± SEM ; n=3; * $p < 0.05$. Statistically significant difference in comparison with Standard group.

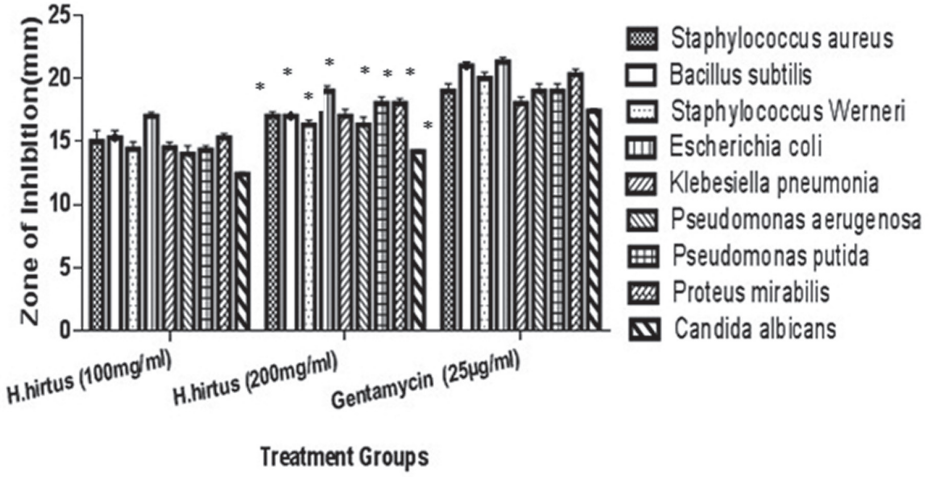

FIGURE 5 - Zone of inhibition of *Hibiscus hirtus* ethanolic extract.

main objective of wound healing is to heal the injury in the shortest time with minimal pain and discomfort to the patient. At the wound site, a flexible, fine scar with maximal tensile strength is desired. With the prepared ointments the wound was treated topically and observed that after the 4th day, dead tissue was removed. The swelling and redness was minimized which indicates that the prepared ointment has tissue debrideeffect at the wound site after 8 days. "Kalka" (application of paste) is a major dosage form for wound healing located in muscle and included slough, according to Acharya Sushruta. Both functions like "Shodhana" (cleaning) and "Ropana" (healing) were made by the paste in DushtaVrana (Ajmeer *et al.,* 2014). Kashaya rasa (astringent) which provides Lekhana (scraping) that helps to slough out necrosed tissue and preparing the wound for healing and helps to stop discharge from the wound. Madhura Rasa (sweet taste) gives nutrition to the wound tissue, which support in granulation tissue development (Dudhamal, Gupta, Bhuyan, 2010). Treatment of *HH* extracts on wounded animals produce significant wound healing activity.

The wound healing is a complex process that involves the synchronization and activation of coagulatory and inflammatory events, epithelialization, fibrous tissue accretion, deposition of collagen, wound contraction, tissue granulation and remodeling (Ghosh, Gaba, 2013) Healing process occurs by immunological activities of victim itself, but various risk factors such as infection and week immunity may cause delay in healing has brought attention to promote this process (Kumar *et al.,* 2008;

Nayak, Anderson, Pereira, 2007). All the parameters that are discussed were significantly affecting wound healing activity of the whole plant. A significant antimicrobial activity has been observed. The components present in the crude extract are responsible for the significant antimicrobial activity. The contraction of the Wound indicates the reduction rate of the unhealed area during the process of healing. Thus, a fast wound contraction rate indicates better efficacy of medication.

Wound contraction plays an significant role in the full thickness wounds closure, where the surrounding skin is pulled in by forces that develop inside the granulation tissue (Ganeshkumar *et al.,* 2012) Significant wound healing activity was produced by *HH* treatment, which may be due to its angiogenic and mitogenic potential. Its prohealing activity was marked, as all the important parameters observed were affected significantly.

The healing tissue requires synthesis of collagen, which is a constituent of growing of the cell. Hydroxyproline concentration is a measure of collagen concentration. The faster rate of healing of the wound is due to higher concentration of hydroxyproline. Biochemical analysis revealed that increased hydroxyproline content, which is a reflection of raised proliferation of the cells and there by collagen synthesis gets increased (Trabucchi *et al.,* 1985; Shukla, Rasik, Dhawan, 1999) In wound healing, type I collagen gene expression is found in every phase of the repair process (Mäkelä, Vuorio, 1985). The synthesis of collagen coincides with increased wound-breaking strength (Viljanto, Ojansuu, Keyworth, 1964). Ultimately, in wound healing, the rather acellular but fiber-rich scar tissue contains, predominantly, fibrils derived from collagen type I molecules (Cohen *et al.,* 1992). Collagen type I can directly cause the migration and adhesion of numerous cell types, including keratinocytes and fibroblasts (Scharffetter-Kochanek *et al.,* 1992). Increased hexosamine content reflects the stabilization of collagen molecules by enhancing electrostatic and ionic interactions (Nayak *et al.,* 2009). Giving strength ,integrity to the tissue matrix and plays an important role in homeostasis and in epithelialization at the latter phase of healing are attributed to collagen (Süntar *et al.,* 2010)

Increased synthesis of hexosamine and hydroxyproline -treated rabbits provide strength to repair tissue and also healing pattern. The result indicates the potent wound healing capacity, which is evident from the contraction of the wound. Increased biochemical parameters and increased tensile strength in healing tissue have thus validated the ethnotherapeutic claim. The study revealed the effect of the *HH* extract on collagen synthesis and their modulatory role in Col 1 a (I) gene expression which is a significant factor contributing to the normal wound healing process. *HH* extract exhibited promising and somewhat better wound healing promoting activity similar to that of the standard povidone iodine ointment.

The study indicates a clear insight about the biochemical mechanisms responsible for the *HH* extract wound healing activity by using a rabbit model along with the significant antimicrobial activity provided pharmacological evidence to the ethnomedicinal claim. Preliminary phytochemical screening the of ethanolic extract of *HH* revealed the presence of alkaloids, flavonoids, tannins, saponins, and carbohydrates. Broad spectrum of antibacterial activity was obtained by extracts of *HH*, which seemed to have beneficial effects on wound healing. *HH* was found to show antibacterial activity against *Escherichia coli, Proteus mirabilis, Bacillus subtilis, Pseudomonas putida, Klebesiella pneumonia, Staphylococcus aureus, Staphylococcus werneri, Pseudomonas aeruginosa* and antifungal activity against *Candida albicans*.

Antimicrobial activity is one of the mechanisms by which some bioactive substance's effect wound healing, hence the investigation of the antibacterial potential of the extract in relation to gentamicin. Most infections on wounds are typically caused by common body bacteria flora (Gbedema *et al.,* 2010). Pseudomonas aeroginosa and Echerichia coli, and Staphylococcus aureus were selected as they comprise the bacteria that commonly colonize open wounds to cause poor healing .The benefits of antimicrobials in wound management have been established in numerous studies suggesting accelerated healing with either systemic or topical application of antibiotics (Langford, Artemi, Benrimoj, 1997), thus, endorse antimicrobial activity as a mechanism of wound healing (Ofori-Kwakye *et al*. 2009; Mbosso *et al.,* 2008; Mensah *et al.,* 2006). The extract has an antibacterial effect against the selected organisms in this study suggesting that the wound healing activity of HH extract may be by an antibacterial activity mechanism.

Phytochemical tests are important in identifying new sources of therapeutically and industrially valuable compound having significance, to make the best use of natural wealth available. The results indicated that the extract contained alkaloids, anthocyanins, flavonoids, saponins, and tannins which are the main phytochemical groups with biological activities. Anthocyanins compounds have the healing properties. The anthocyanins have been found to be cardioprotective, hypocholesterolemic; antioxidative and hepatoprotective (Jonadet *et al.,* 1990). They also have an antioxidant activity and inhibit low density lipoprotein (LDL) oxidation (Del Rio *et al.,*

2013). Alkaloids also interfere with cell division; hence the presence of alkaloids in the plant makes it a possible remedy in the treatment of cancer. Flavonoids are well known for their anti-viral, anti-inflammatory, antioxidant activity, cytotoxic and also used in the treatment of hypertension, diabetes, rheumatic fever (Usoh *et al.,* 2005; Tolulope, 2007). In the present study, polyphenols were detected. Polyphenols have attracted a great attention in relation to their potential for beneficial effects on health. By blocking key enzymes at microbial metabolism tannins decrease the bacterial proliferation. It is clear that extract possesses good phytoconstituents that will be helpful in the future for the cure of different types of diseases.

CONCLUSION

Wound healing activity of our ethanolic extract of *HH* has shown the good effect which has proved by different physical, histological, biochemical parameters. Even our plant has shown significant antimicrobial activity. It may be due to the presence of major active constituents present in our plant. From our preliminary studies we have confirmed that the activity is due to the presence of anthocyanins. Further studies are required to confirm the main active constituents responsible for the activity. The above results could justify the inclusion of the plant in the wound healing management in folk medicine.

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