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Comparative study of wound-healing effect of bark extracts of *Ficus religiosa* & *Ficus benghalensis* by mice model

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Abstract
The aim of the study was the comparative study of the wound healing potential of the bark of *Ficus religiosa* and Ficus benghalensis. The locally available plants like *Ficus religiosa* and Ficus bengalensis have the capability to cure diseases traditionally since long years, due to this reasons these two plants were selected for the study. It was found that ethanol and hydroalcoholic extraction of two plants that is *Ficus religiosa* and *Ficus benghalensis* showed high wound healing activity. The plant extracts were used to study their phytochemical compositions i.e. total phenols contents, flavonoids contents, terpenoids content and proteins contents. Most of the biologically active phytochemical presents in the ethanol extract. The results obtained that Wound healing potential of the bark of *Ficus religiosa* is higher than the bark of Ficus benghalensis. The antimicrobial activity was evaluated by well diffusion method while wound healing effect in rats was evaluated using the *in vitro* and *in vivo* wound model infected. In case of antimicrobial activity the ethanolic extract of *Ficus religiosa* showed better antimicrobial activity. The ethanolic and hydroalcoholic extract of bark of two plant that is *Ficus religiosa* and *Ficus benghalensis* compared for *in vitro* wound healing activity. For *in vitro* Wound healing activities including Inhibition of RBC haemolysis was performed. In Inhibition of RBC haemolysis, when the decrease in absorbance at 517nm it increases the RBC membrane stabilization activity of the plants sample. For RBC membrane stabilization experiment Soframycin taken as the standard solution and for *in vitro* wound healing activity using excision wound model was performed. In both case *in vivo* and *in vitro* wound healing model ethanolic extract of *Ficus religiosa* having higher wound healing activity as compared to hydroalcoholic extract.

Keywords: Wound, wound healing, *Ficus religiosa*, *Ficus benghalensis*

Introduction
Wounds are inescapable events of life, which arise due to physical or chemical injury or microbial infections. The healing of wounds often deviates from normal course, under-healing, over-healing or failure of wounds [1]. Wound healing consists of a complex, well-organized cascade of biochemical and cellular events that involves tissue repairs and regeneration [2-3]. It is fundamentally a connective tissue response and involves the activity of an intricate network of blood cells, cytokines and growth factors, which ultimately leads to the restoration of the injured skin or tissue to normal condition [4-5]. The aim of wound care, which must occur in a physiologic environment conducive to tissue repair and regeneration, is to promote healing in the shortest time possible, exclude secondary infections and minimize pain, discomfort and scarring [6]. The entire process of wound healing, which begins at the moment of injury and may continue for prolonged period, can be grouped into three distinct phases, namely: Inflammatory, proliferative and remodeling phase, each of these phases is characterized by a series of events [7]. These processes of healing are known to be influenced by several factors such as infections, nutrition, drugs and hormones, type and sites of wound, and certain disease conditions [8]. Many medicinal plants have a very important role in the process of wound healing. Plants are potent healers because they promote the repair mechanisms in the natural way. More than 70% of wound healing Pharma products are plant based. The plant base materials are used as first aid – antiseptic coagulants and wound wash. Medicinal plants are coming into prominence because of the over-use of conventional medicines such as antibiotics which has resulted in the development of resistance in many infectious organisms. Thus, herbal preparations can be more effective than conventional medicines and their non- toxic nature means that they can be administered over long periods. In recent times, focus on plant research has increased all over the world and large body of evidence has been collected to show immense potential of medicinal plants used in various traditional systems.

More than 13,000 plants have been studied during the last 5-year period [9]. A number medicinal plants such as *Ficus religiosa, Ficus benghalensis, Morinda citrifolia, Cassia alata, Jatropha curcas, Tridax procumbens, Wrightia tinctoria, Trigonella foenumgraceum* among others have been identified and employed in folk medicine for wound care [10-17]. *Ficus religiosa* L. is an important medicinal tree species belonging to the family Moraceae. It is a large widely branched tree with leathery, heart shaped, long tipped leaves on long slender petioles and purple fruits growing in pairs [18-20]. Bark of *Ficus religiosa* occurs in flat or slightly curved pieces, varying from 1.0 - 2.5 cm or more in thickness; outer surface brown or ash colored; surface uneven due to exfoliation of cork; inner surface smooth and somewhat brownish; fracture - fibrous; taste – astringent [21]. It is a large perennial tree, glabrous when young, found throughout the plains of India upto 170 m altitude in the Himalayas and is one of the most revered trees in Asia. The specific term *religiosa* is related to the religious significance attached to this tree. In medicinal field, *Ficus religiosa* is gaining great attention because it has many compounds which are beneficial in treatment of many diseases like diabetes, skin diseases, respiratory disorders, central nervous system disorder, gastric problems etc. [22-23]. Six parts of the trees (i.e., seeds, bark, leaves, fruit, latex and roots) are valued for their medicinal qualities. The bark is used for the treatment of various skin diseases, inflammations and glandular swelling of the neck, scabies, in ulcers, as astringent, as tonic. Its root bark is useful for stomatitis, clean ulcers, and promotes granulations. The ethanol bark extract of *Ficus religiosa* was reported to possess wound healing [24]. Moreover, the barks of *Ficus religiosa* are an important ingredient in many Ayurvedic formulations, such as Nalpamaradi tailam, Chandanasavam, Nyagrodhadi churna and Saribadyasavam [25-26].

Ficus benghalensis (Moraceae, Mulberry family) is usually identified as Banyan tree or Vata or Vada tree in Ayurveda. It is endemic to Bangladesh, India and Sri Lanka. It is also known as Bengal fig, Indian fig and East Indian fig, Indian Banyan or simply Banyan (English), also borh, nyagrodha (Sanskrit), Bat, Bargad and Bar (Hindi). Ficus means fig and bengalensis means belonging to or is of Bengal [27]. A very large tree up to 30 m in height with widely spreading branches bearing many aerial roots functioning as prop roots, bark greenish white, leaves simple, alternate, often in clusters at ends of branches, stipulate,10 to 20 cm long and 5 to 12.5 cm broad, broadly elliptic to ovate, entire, strongly 3 to 7 ribbed from the base; the fruit rescales are axillary, sessile, in pairs, globose, brick red when ripe, enclosing male, female and gall flowers; fruits small, crustaceous achenes, enclosed in the common fleshy receptacles [28]. Bark smooth grey hard surface and uneven 0.5-1.9 cm thick, on rubbing white papery flakes come out from the outer surface inner surface light brown fracture fibrous taste mucilaginous without any characteristics odour [29-30]. The bark is astringent and tonic and used in diabetes and leucorrhoea, lumbago, sores, ulcers pains and bruises [31]. It is used in traditional system of medicine like Ayurvedic, Siddha, Unani and homoeopathy. Some important Ayurvedic marketed formulations formulated from *Ficus bengalensis* are Nyagrodhadi churnam (Bhaishajya Rutnavali), Saarivaadya Chandanaasava, Dineshavalyaadi Taila (Sahasrayoga) [32].

Material Methods

Sample collection and Authentication

The bark of *Ficus religiosa* and *Ficus benghalensis* were collected from the Janjgir district, Chhattisgarh, and allowed to sun dry and was authenticated by Mr. Rajesh Panday in Dravyaguna Vigyan, Govt. Ayurvedic College, Raipur (C.G.).

Study of Pharmacognostic characteristics of bark

Macroscopic characterization

The macroscopic characters of the bark of *Ficus religiosa* and *Ficus benghalensis* such as colour, odour, taste, size, shape etc. were evaluated.

Microscopic evaluation

The T.S. of the bark of *Ficus religiosa* and *Ficus benghalensis* was prepared and observed in microscope.

Physicochemical evaluation

For evaluation of physicochemical characters of bark of *Ficus religiosa* and *Ficus benghalensis* extractive value. Ash values of crude drug, acid insoluble ash value, and loss on drying was determined by standard methods.

Phytochemical screening of bark

Phytochemical screening of bark of *Ficus religiosa* and *Ficus benghalensis* was done to detect alkaloids, carbohydrates, glycosides, steroids, terpenoids, saponins, phenols, proteins, amino acids and flavonoids.

Extraction

Extraction was done by maceration process. Maceration process involves separation of medicinally active portion of crude drugs. It is based on the immersion of crude drugs in a bulk of solvent or menstruum. Ethanolic and hydroalcoholic extract were used for the extraction of bark of *Ficus religiosa* and *Ficus benghalensis.* The dried bark of *Ficus religiosa* and *Ficus benghalensis* was taken in a stoppered container with about 750ml of menstruum (ethanolic and hydroalcoholic) and allowed to stand for at least 3-7 days with frequent shaking. The mixture of crude drug containing solvent was filtered until most of the liquid drain off and then evaporates.

Phytochemical analysis of extracted bark

Total phenolic content

After Crude ethanol extracts (0.5mg/ml) preparation, 1ml of extract mixed thoroughly with 1ml of Folin-Ciocalteu reagent and 0.8ml of $NaCO_3$(7.5%). The mixture was allowed to stand for 30 min at room temperature with intermittent shaking. Absorbance was measured at 765 nm. The concentration of the total phenolic compounds in extract was determined as gram of Gallic acid equivalent.

Total flavonoid content

The method based on the formation of complex flavonoid aluminium, having absorbance maximum at 430 nm. Quarcetin was used to make a calibration curve. 0.5 ml of extract solution was mixed with 2ml distilled water and 0.15 ml of 15% $NaNO_2$. The mixture was incubated for 6 min. and then 0.15 ml of 10% $AlCl_3$ was added. After 6 min, 2ml of 4% NaOH solution was added. Distilled water was added to bring the sample to 5ml. before measuring the absorbance at 510nm. The mixture was allowed to stand for 15 min. The flavonoids content was expressed as quarcetin equivalent in mg/g extract.

Antimicrobial activity

Agar well diffusion method was used. Petriplates containing 20ml nutrient Agar medium were seeded with 24hr culture of

bacterial strains (*Staphylococcus aureus, Streptococcus mutans, Bacillus coagulans* bacteria) and PDA plates were seeded with *Candida albicans* fungi. Wells were cut and 20 µl of the plant extracts (namely ethanolic and hydroalcoholic extracts) were added. The nutrient agar plates were then incubated at 37°C for 24 hours and PDA plates were incubated at 25^0C for 2-3 days. The antimicrobial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Soframycin was used as a positive control.

***In vitro* wound healing model**

RBC membrane stabilization or Inhibition of RBC haemolysis

The blood was collected from animal, mixed with equal volume of Alsever's solution (2% dextrose, 0.7% sodium citrate, 0.5% citric acid and 0.4% sodium chloride). The obtained solution was washed with saline three times. RBC layer was collected and diluted with phosphate buffer saline to make 10%v/v solution. 100µL of 10% RBC solution was heated at 56◦C for 30 min followed by centrifugation at 2000 rpm for 8-10 min at room temperature. Clear supernatant was collected and absorbance was recorded at 560 nm. % haemolysis inhibition was calculated by following formula:

$$\% \text{ haemolysis inhibition} = 100-\{[1-(\frac{Blank\ Sample}{Blank})] \text{ x}100\}$$

***In vivo* wound healing model**

Preparation of Topical Formulation

The topical gel (10% w/v) was prepared by soaking sodium CMC in water for 30 min. 200mg of *Ficus religiosa* and *Ficus benghalensis* extract was incorporated in 100 gm of sodium carboxymethyl cellulose gel by mixing so as to get 10% w/w of EEFR and EEFB gel. Methyl paraben was incorporated in the both EEFR and EERB gel as preservative.

Experimental design

The *In vivo* studies were conducted by the approval of Institutional Animal ethical Committee, SRIP Kumhari. Animals were wounded under light ether anesthesia, semi-aseptically. The animals were assigned in to six groups, each group containing six animals. First group was untreated group which was taken as control (Group- A). In Second group (Group-B) wounds received topical application of ethanolic extract of bark of *Ficus religiosa* gel. In Third group (Group-C) animals were applied ethanolic extract of *Ficus benghalensis* gel. In fourth group (group D) wound received topical application of hydroalcoholic extract of bark of *Ficus religiosa* gel. In fifth group (group E) wound received topical application of hydroalcoholic extract of bark of *Ficus benghalensis* gel and animals in Group- F (Reference Standard) received treatment of Framycetine Sulphate Cream (FSC) in excision wound. No other topical or systemic therapy was given to animals during the course of this study. The experimental protocols were approved by the Institute Animal Ethical Committee. Wounded animals were kept separately, one in each cage.

Excision wounds

The rats were anaesthetized prior to creation of the wounds by inhalation with diethyl ether. The dorsal fur of the animal was shaved with razor blade and the area of the wound to be created was outlined on the back of the animals with methylene blue using a round seal 1cm diameter. A full thickness of the excision wound of 1.0 cm in width (circular area 4.90 cm2) and 0.2 cm depth was created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open. All the surgical interventions were carried out under sterile condition. Treatment was continued till the complete healing of wound. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. Contractions contributed for wound closure on 4th, 8th, 12th, 16th and 20th post-wounding day were observed along with period of epithelisation.

Result

Macroscopic evaluation

Bark of *Ficus religiosa* was flat or slightly curved pieces, varying from 1.0 - 2.5 cm or more in thickness; outer surface brown or ash colored; surface uneven due to exfoliation of cork; inner surface smooth and somewhat brownish; fracture - fibrous; taste – astringent; while bark of *Ficus bengalensis* was smooth grey hard surface and uneven 0.5-1.9 cm thick, on rubbing white papery flakes come out from the outer surface inner surface light brown fracture fibrous taste mucilaginous without any characteristics odour.

Microscopic evaluation

On microscopic evaluation of the *Ficus religiosa* the cortex was fairly wide and composed of several rows of cells and contained several, scattered, one to few groups of stone cells. The stone cells were oblong to rectangular, spherical or polygonal and had thick, pitted walls. The cortical parenchyma cells were thin-walled and more or less cubical to oblong. Several of them were loaded with compound starch grains. The two seriate modularly rays were present in cortex region; while the T.S. of the cortex region of the bark of *Ficus benghalensis* showed the compactly arranged parenchymatous cell between which multiseriate medullary rays were present with simple pits. The cortex contained several, scattered, one to few groups of stone cells. (Fig. 1 and 2).

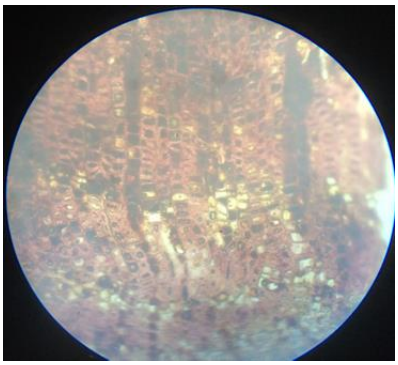

Fig 1: T.S. of *Ficus religiosa*

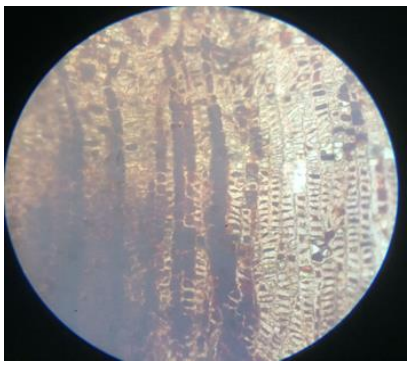

Fig 2: T.S. of *Ficus benghalensis*

Physicochemical evaluation

Extractive value was recorded higher in bark of *Ficus religiosa* as compared to *Ficus benghalensis;* while ash value was highest in bark of *Ficus benghalensis.* In case of Acid insoluble ash value again *Ficus religiosa* bark showed more than bark of *Ficus benghalensis.* LOD was found equal in both barks. (Table 1).

Table 1: Physiochemical Evaluation of bark of *Ficus religiosa* and *Ficus benghalensis*

S. No.	Plant	Extractive value		Ash Value	Acid soluble ash value	Loss on drying (LOD)
		Ethanolic	Hydro-alcoholic			
1.	*Ficus religiosa*	9.6% w/w	26.4% w/w	5% w/w	40% w/w	0.84% w/w
2.	*Ficus benghalensis*	9.2% w/w	25.6% w/w	7.5% w/w	13% w/w	0.84% w/w

Note: The values were average of 3 determination.

Phytochemical screening of barks

In both *Ficus religiosa* and *Ficus benghalensis* bark tannins, saponins, flavonoids, terpenoids and phenols were present but steroids were observed only in *Ficus religiosa* bark. In both, cardiac glycosides, carbohydrates, Proteins and alkaloids were absent. (Table 2)

Table 2: Phytochemical screening of barks

S. No.	Phytochemical	*Ficus religiosa* (Bark)	*Ficus benghalensis* (Bark)
1.	Tannins	+	+
2.	Saponins	+	+
3.	Flavonoids	+	+
4.	Cardiac glycosides	-	-
5.	Steroids	+	-
6.	Terpenoids	+	+
7.	Carbohydrates	-	-
8.	Phenols	+	+
9.	Proteins	-	-
10.	Alkaloids	-	-

Note: (-) Absence (+) Present

Phytochemical Analysis of barks after extraction

Phenolic content was found higher in ethanolic extract of both *Ficus religiosa* and *Ficus benghalensis* as compare to hydroalcoholic extract. Ethanolic and hydroalcoholic extract of *Ficus benghalensis* having more phenolic content as compare to *Ficus religiosa.* The ethanolic extract of both *Ficus religiosa* and *Ficus benghalensis* having more flavonoid content *i.e.* 79.50 µg/10mg and 80.21 µg/10mg as compare to hydroalcoholic extract. Ethanolic and hydroalcoholic extract of *Ficus benghalensis* having more flavonoid content as compare to *Ficus religiosa.* (Table: 3).

Table 3: Phytochemical Analysis of barks after extraction

S. No.	Plant (Bark)	Phenolic content		Flavonoid content	
		Ethanolic	Hydro alcoholic	Ethanolic	Hydro alcoholic
1.	*Ficus religiosa*	143.54 µg/10mg	123.12 µg/10mg	79.50 µg/10mg	71.67 µg/10mg
2.	*Ficus benghalensis*	146.35 µg/10mg	135.85 µg/10mg	80.21 µg/10mg	76.14 µg/10mg

Note: The values were average of 3 determination.

Antimicrobial activity of barks

The ethanolic extract of *Ficus religiosa* (EFR) showed high antibacterial activity against *Staphylococcus aureus, Streptococcus mutans, Bacillus coagulans* bacteria and *Candida albicans* fungi as compared to ethanolic extract of *Ficus benghalensis* (EFB).

The hydroalcoholic extract of *Ficus benghalensis* (HEFB) showed high antibacterial activity against *Staphylococcus aureus*, *Bacillus coagulans* bacteria and *Candida albicans* fungi as compair to hydroalcoholic extract of *Ficus religiosa* (HEFR) but hydroalcoholic extract of *Ficus religiosa* high antibacterial activity against *Streptococcus mutans* bacteria as compair to hydroalcoholic extract of *Ficus benghalensis.*

Ficus religiosa showed high antimicrobial activity as compare to *Ficus benghalensis* in case of ethanolic extract but in case of hydroalcoholic extract, *Ficus religiosa* showed highest growth inhibition against *Streptococcus mutans* and *Bacillus coagulans* while against *Staphylococcus aureus* and *Candida albicans*, *Ficus benghalensis* showed highest growth inhibition. (Table: 4).

Table 4: Antimicrobial activity of barks

Sample	Mean zone of growth inhibition (mm ± SD)			
	Staphylococcus aureus	Streptococcus Mutans	Bacillus coagulans	Candida albicans
HEFR	1.41±0.29	1.56±0.15	1.63±0.12	1.51±0.02
HEFB	1.51±0.23	1.53±0.32	1.60±0.35	1.78±0.028
EFB	1.76±0.53	1.45±0.23	1.33±0.12	1.65±0.05
EFR	1.60±0.27	1.71±0.33	1.91±0.40	1.9±0.10
F_{cal}	0.524	0.495	2.153	23.27
F_{tab}	0.674	0.691	0.178	0.002
CD (0.05%)	0.00	0.00	0.00	0.11
F_{test}	NS	NS	NS	S
S.Ed(±)	0.148	0.110	0.238	0.165

Note: The values were average of 3 determination.

***In vitro* wound healing model**

The ethanolic extracts of both *Ficus religiosa* and *Ficus benghalensis* having more RBC membrane stabilization activity when compared to hydroalcoholic extracts. Both Ethanolic and hydroalcoholic extracts of *Ficus religiosa* showed slightly higher RBC membrane stabilization activity in comparison with *Ficus benghalensis.* Higher RBC membrane stabilization was recorded with 2.0 mg/ml in both ethanolic and hydroalchoholic extracts of *Ficus religiosa i.e.* 90.84% and 90.31% respectively. (Table 5)

Table 5: *In vitro* wound healing activity

S. No.	Concentration (mg/ml)	%Haemolysis inhibition			
		Ethanolic		Hydro alcoholic	
		Ficus benghalensis	***Ficus religiosa***	***Ficus benghalensis***	***Ficus religiosa***
1.	0.5	45.98 %	49.99 %	34.28 %	39.00 %
2.	1.0	55.95 %	58.08 %	41.12 %	54.17 %
3.	1.5	67.65 %	77.69 %	58.03 %	67.70 %
4.	2.0	90.79 %	90.84 %	77.78 %	90.31 %

***In vivo* wound healing model**

Excision model

In the excision wound study the wounds were treated with soframycin cream and ethanolic and hydroalcoholic extract of bark of *Ficus religiosa* and *Ficus benghalensis* gel. The treated animals showed significant reduction in wound size was on 2th day, on 4th day on 8th, on 12th, on 16th, on 18th, on 20th day when compared to control. Complete healing was observed in day 17. The results of these groups were compared with the healing activity of untreated group which took more than 20 days for wound closure and fall of eschar. But there was no significant difference between test and standard. (Fig 3 and 4; Table: 6).

Table 6: *In vivo* wound healing activity

Parameter	Wound area (mm) and percentage of wound concentration				
Post wounding days	**Control**	**HAEFR**	**EEFR**	**HAEFB**	**EEFB**
Day 0	503.66 ±2.9857	503 ±3.2151	505.66 ±2.1554	503 ±3.2151	505.66 ±2.1554
Day 2	478.33 ±3.7749 (5.02%)	469.666 ±1.202 (6.62%)	465.33 ±1.3335 (7.97%)	470.690 ±1.205 (6.62%)	468.39 ±1.3340 (7.97%)
Day4	425.33 ±1.3336 (15.55%)	393.33 ±0.989 (21.80%)	366 ±2.7814 (27.61%)	395.33 ±0.999 (21.80%)	369 ±2.7901 (27.61%)
Day8	338 ±1.1549 (32.89%)	292.66 ±1.3335 (41.81%)	268.66 ±1.7641 (46.86%)	295.66 ±1.3337 (41.81%)	271.66 ±1.7652 (46.86%)
Day12	211.66 ±1.202 (57.97%)	165 ±1.5278 (67.19%)	125 ±1.3418 (75.27%)	168 ±1.5282 (67.19%)	129 ±1.3423 (75.27%)
Day16	77.33 ±1.7641 (84.64%)	22 ±1.1549 (95.62%)	13.66 ±1.202 (97.29%)	25 ±1.1552 (95.62%)	16.63 ±1.206 (97.29%)
Day18	24.33 ±1.202 (95.16%)	5.66 ±0.9546 (98.87%)	00 ±00 (100%)	7.25 ±0.9549 (98.87%)	00 ±00 (100%)
Day20	9 ±0.8565 (98.21%)	00 ± 00 (100%)	00 ±00 (100%)	00 ± 00 (100%)	00 ±00 (100%)
Period of epithelialization (day)	21.5 ±0.3416	18.33 ±0.2108	17.16 ± 0.1666	18.37 ±0.2112	17.19 ±0.1670

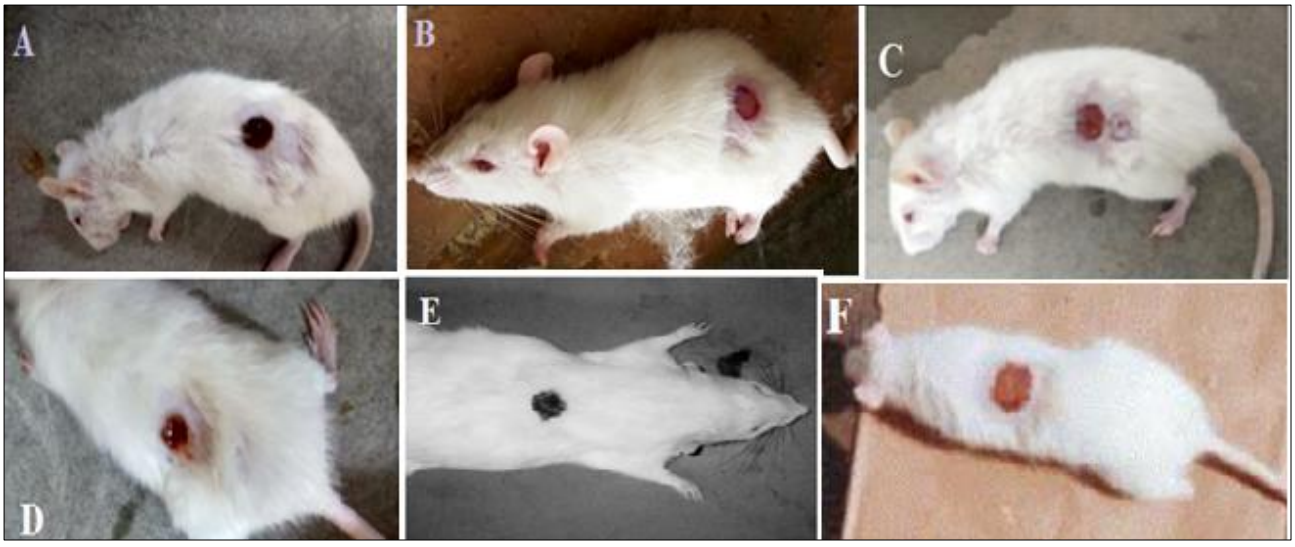

Fig 3: Excision wound on-0 day

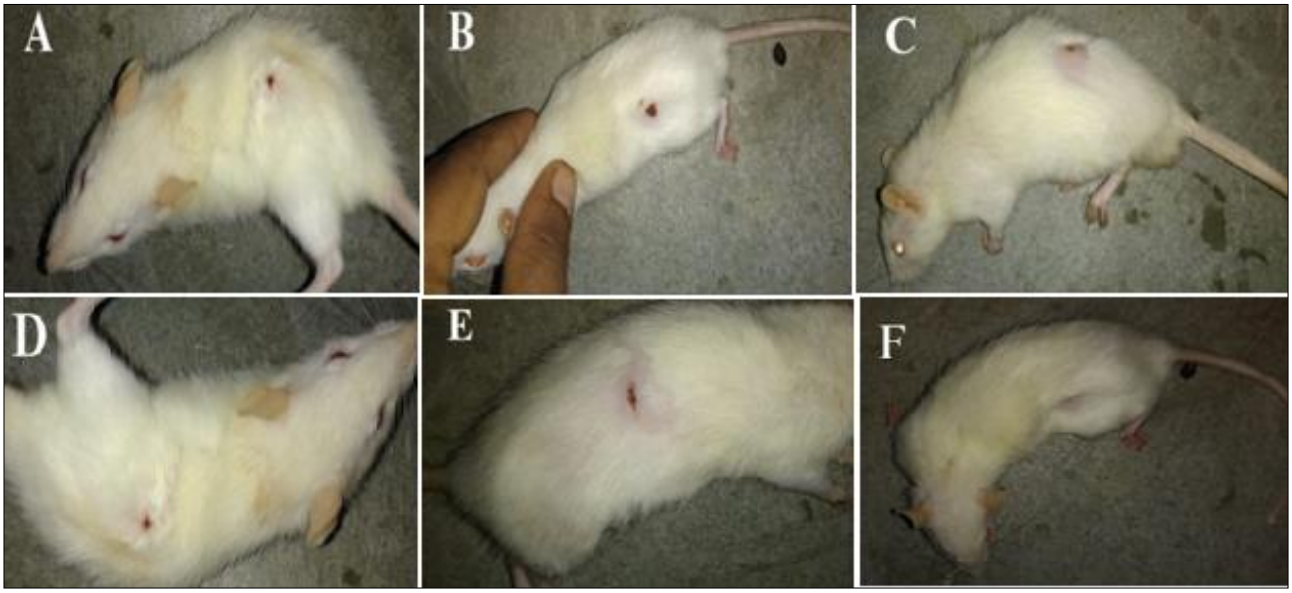

Fig 4: Excision wound on-17 day

Discussion

The present study was carried out to compare the wound healing potential of extracts of barks of *Ficus religiosa* and *Ficus benghalensis*. In phytochemical analysis, both plant barks showed presence of tannins, saponins, flavonoids, terpenoids and phenols. The presence of higher amounts of phenols in the plants belonging to the family Moraceae, particularly in the genus *Ficus* is reported earlier by Ao C, *et al.*, 2007 [33]. Phenolic compounds are considered as the most important antioxidative components of plant material because of the positive correlation between the concentration of plant phenolics and its total antioxidant capacity [34]. It is also reported earlier that antioxidant nature of the flavonoids of *Ficus religiosa* is responsible for wound healing activity. Flavonoids and triterpenoids have astringent and antimicrobial property which promotes the wound-healing process via wound contraction and increased rate of epithelialization [35-37]. Tannins are active detoxifying agents and also inhibit bacterial growth. Earlier Goren *et al*., 1996 [38] also demonstrated the therapeutic value of phytochemical extracts of Ficus species. Phytochemical responsible for wound healing in medicinal plants is also reviewed by Ghosh and Gaba (2013) [39].

Conclusion

In this present study the ethanolic extract of *Ficus religiosa* is found to be stronger in displaying the abilities of wound healing as compare than other extract (hydroalcoholic extracts of *Ficus religiosa* and hydroalcoholic extracts of *Ficus benghalensis*.

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