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Herbal antimicrobial gel with leaf extract of *Cassia alata* L.

Parbin Iraqui[1], Tapash Chakraborty[2], Malay K. Das[2]*, R.N.S Yadav[1]

[1] Department of Life Sciences, Dibrugarh University, Dibrugarh 786004 INDIA

[2] Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh 786004 INDIA

ABSTRACT

Topical application of antimicrobials at the site of infection offer greater advantages as compared to systemic therapy. The present study reports for the first time, the *in vivo* wound healing potential of an herbal antimicrobial gel containing pure bioactive leaf extract of *Cassia alata* L. The methanolic leaf extract exhibited significant antibacterial and antifungal activity against the tested bacteria (*Staphylococcus aureus* MTCC 9542) and fungi (*Candida albicans* MTCC 4842) due to the presence of alcohol and ketone containing bioactive moieties. A 1% (w/w) bioactive leaf extract based-hydrogel was formulated and evaluated for its wound healing potential in rat model with surgical site infection in the dorsal area. This herbal gel significantly enhanced the wound healing as assessed by the contraction of wound length and bio burden characteristics compared to the marketed antimicrobial formulations. The formulated herbal gel could find use as very promising and innovative topical alternative for the treatment of skin infections caused by bacteria as well as fungal strains without hazard to human health based on the fact of its traditional use by the Assamese people with no toxic effects.

Keywords: *Cassia alata* L.; methanolic leaf extracts; antimicrobial gel; antibacterial; antifungal; wound healing.

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***Address for Correspondence:**

Malay K. Das, Drug Delivery Research Laboratory, Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh 786004 INDIA

INTRODUCTION

Infectious diseases are increasing day by day due to various reasons like climate change, antibiotic resistance and microbial adaptation. *Staphylococcus aureus, Staphylococcus epidermidis, Salmonella typhi, Mycobacterium tuberculosis, Vibrio cholerae, Escherichia coli, Candida albicans, Mycosporum canis, Trichophyton rubrum* are the causative agents that cause infectious diseases like pneumonia, endocarditis, septic arthritis, cholera, typhoid, tuberculosis, cellulitis, ringworm, impetigo. To treat these infections both synthetic and herbal medicines have been used by the people for a long time[1]. Nowadays, herbal medicines are gaining importance and are used as an alternative to synthetic drugs, as these drugs have no or least side effects[2]. Medicinal plants like Neem, Turmeric, Garlic, Tulsi, Aloe vera have been used to treat infectious diseases for a long time[3-8]. These medicinal plants are also useful in skin infections[9].

The North Eastern region of India is blessed with biological diversity as numerous medicinally important plants and herbs are available here. These medicinal plants have been used for the treatment of various infectious diseases by its local people from ancient times[10-12]. Due to the indiscriminate use of the antimicrobial drugs, developments of drug-resistance by microbes have also been frequently reported[13]. Hence herbal formulations for topical use, for treating skin diseases became popular. As the development of bacterial resistance to antibiotics and other synthetic antimicrobials, scientists found literally thousands of phytochemicals from plants which inhibit different types of microorganisms with different mechanisms and which are safe and broad spectrum antimicrobials in the treatment of resistant microbial strains[14]. Topical application of antimicrobial agent at the site of infection offer greater advantages as compared to systemic therapy[15-18]. Firstly, the required concentration for antimicrobial activity of the drug at the target site can be easily achieved after topical dosing. Secondly, topical administration results in much lower or almost undetectable systemic levels of the active constituents[19]. Thirdly, it can avoid an unnecessary exposure of the gut flora to the antimicrobial agents which may lead to drug-resistance or depletion of the natural bacterial flora of the GIT. Therefore, topical application of antimicrobial agents is considered an important alternative to the systemic delivery of drugs for the treatment of skin diseases[17].

Cassia alata L. is a shrub of about 3–4 m tall, with leaves 50–80 cm long (Figure 1). Though the plant is native to Mexico, it can be found in diverse habitats throughout the world, including North East India. The leaves of this plant are traditionally used by the people of Assam in skin diseases,

especially in ringworm. This plant has antibacterial, antifungal and antioxidant properties[20].

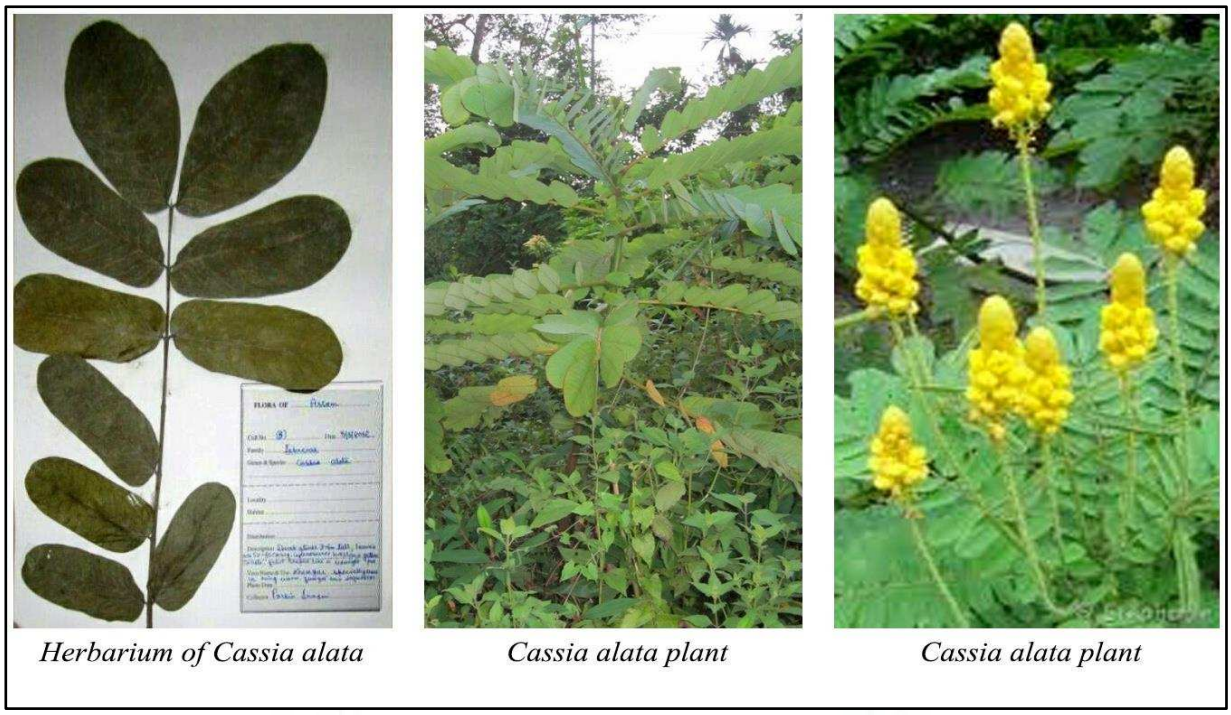


Figure 1: Herbarium and pictures of *Cassia alata* L. plant.

Cassia alata L. plant has been extensively studied for its phytochemicals and antimicrobial activities as reported elsewhere[21-26]. The whole plant possesses different types of active constituents which have been shown to have a wide spectrum of activities. The stem bark extract of *Cassia alata* L. has been reported to contain 29 different bioactive compounds including flavonoids, phenol, carotenoids, alkaloids, steroids, saponins[22-24]. Leaf extracts with different solvent systems showed antimicrobial properties against different Gram positive and Gram negative bacteria including different fungus[21,25-26]. These diverse activities as shown by the leaf extracts of *Cassia alata* L. is mainly because of different types of phytocomponents like phenol, flavonoid, saponins, alkaloid along with various minerals like zinc, copper, selenium, sodium, calcium, potassium, magnesium, cadmium and phosphorus present in it[27-29]. The leaves also contain vitamins like ascorbic acid, riboflavin and niacin[30]. The seed extract of *Cassia alata* L. contains alkaloids having antimicrobial activity against bacteria like *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,* and fungi like *Candida albicans* and *Aspergillus niger*[31]. The root extract of *Cassia alata* L. also contains different phytochemicals like phytosterol, quinone, resin, saponins and tannin in it and is equally effective against fungus, Gram-positive and Gram-negative bacteria[32]. The essential oils of *Cassia alata* L. have also been studied and reported to have various phytocomponents in it. The GC-MS analysis showed the presence of seven compounds in the oil of *Cassia alata.* L which is also having a wide spectrum of antimicrobial properties similar to other aerial parts of the plant[33]. The extract of *Cassia alata* L. has also been reported to show anticancer properties. On treating leukemia cells (LI290) with *Cassia alata* L. extract showed significant reduction in the production of polyamines by the leukemia cells[34]. Cytotoxicity assay against osteosarcoma (MG-63) cell lines showed that the leaf extract of *Cassia alata* L. moderately inhibits cell growth of the MG-63 carcinoma[35].

All the available reports on *Cassia alata* L. deal only with the extraction of the phytochemicals and evaluation of antimicrobial activity *in vitro*[27-29, 31-32]. No reports have yet been published on the clinical or pharmaceutical application of the pure extracts of the plant. The present study reports the development and evaluation of herbal antimicrobial gel containing pure bioactive leaf extract of *Cassia alata.* L.

Hydrogels are a common form of topical application, which possesses swollen three-dimensional networks of hydrophilic polymers held together by association bonds or cohesive forces. This has controlled release applications, because of their soft tissue biocompatibility, the ease with which drugs are dispersed in the matrix and the high degree of control achieved by selecting the physical and chemical properties of the polymer network. Hydrogels achieve sustained release by diffusion from a reservoir through the microporous membrane into the skin[36].

MATERIALS AND METHODS

Materials: Carbopol 934 (C934) and Triethanolamine (TEA) were purchased from Loba Chemie, Mumbai, India. Propylene glycol (PG) and sodium benzoate are obtained from Merck, India. Renicol (Chloramphenicol, 1% w/w; Klar Sehen Pvt. Ltd, Kolkata, West Bengal, India) and Daktarin (Miconazole nitrate, 2% w/w; Janssen-Cilag Pharmaceuticals, Mumbai, Maharashtra, India) were taken as standard antibacterial and antifungal formulation, respectively, for comparison. Pure strains of *Staphylococcus aureus* (MTCC 9542) and *Candida albicans* (MTCC 4842) were purchased from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. All the other chemicals and reagents used were of analytical grade.

Methods:

Collection of plant material and preparation of leaf extract

Cassia alata L. is locally known as Khor gos and its English name is Candle bush. Leaves of *Cassia alata* L. were collected from Jokai Botanical Garden cum Germplasm Center, Dibrugarh. The sample was authenticated at the Department of Life Sciences, Dibrugarh University, Dibrugarh India (DULSc

457). The collected leaves were washed thoroughly with water and dried under shade for seven days. The dried leaves were ground into a fine powder (#40, sieve number 40) using a mechanical grinder and the powder samples were kept in airtight container for further use. Methanolic leaf extract of *Cassia alata* L. was prepared by cold extraction method[37]. Briefly, 10 g of air-dried leaf powder of the plant was mixed with 100 ml of methanol in a conical flask, plugged with cotton wool and kept in the shaker incubator (Scigenics Biotech India Pvt. Ltd. Chennai; Model: LETTD) for 72 hours. Then the mixture was filtered using Whatman filter paper (No.1). The filtrate was concentrated on a water bath (REMI, Mumbai, India; Model: RSB 12) and yield of the residue was determined. The extraction yield was 71.86% w/w. 1 mg of residue was dissolved in 1 ml of Dimethyl sulphoxide (DMSO) to get the concentration of 1mg/ml. The leaf extract was tested for the presence of phytochemicals using the standard methods reported elsewhere[38]. Further, the leaf extract of Cassia *alata* L. was tested for antimicrobial activity against bacterial strains viz. *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and fungal strains viz. *Candida albicans, Microsporum Canis,* and *Trichophyton rubrum* using agar well diffusion method[39].

The methanolic leaf extract of *Cassia alata* .L was subjected to Silica gel (mesh size 120-125 µm Merck Millipore, Bengaluru, India) column chromatography using different solvent systems (hexane, chloroform, ethyl acetate, ethanol, methanol and their mixtures in different ratios)[40]. The various binary solvent systems of hexane:chloroform (8:2, 5:5, 2:8), chloroform:ethyl acetate (8:2, 5:5, 2:2), ethyl acetate:ethanol (8:2, 5:5, 2:8), ethanol:methanol (8:2, 5:5, 2:8) were used in the column chromatography. From the column chromatography, seventeen numbers of fractions were obtained. Out of which, the bioactive fraction with binary mixture of ethyl acetate and ethanol (8:2) was found to have antimicrobial activity against *Staphylococcus aureus* and *Candida albicans* when tested with agar well diffusion method. This bioactive column fraction was then subjected to thin layer chromatography (TLC) to determine the number of components present in the fraction. TLC was performed on pre-coated aluminum TLC plates of silica gel G 0.25mm thick (size: 20×20 cm, Sigma–Aldrich, India) using solvent ethyl acetate and ethanol in the ratio of 6:4. The bioactive fraction was concentrated by evaporation and applied at one end of the plate. The plate was kept in the chamber containing the mixture of ethyl acetate and ethanol. After air drying, spots were visualized by exposing plates to iodine vapors.

This fraction was concentrated to dryness then dissolved in methanol (concentration 1mg/ml) and was outsourced for Preparative High Performance Liquid Chromatography (Preparative HPLC) (HPLC 1260 Infinity, Agilent Technologies, USA) at Biotech park IIT, Guwahati for separation of bioactive components. Chromatograms of two component peaks with retention times in the range of 1.5 – 4.5 minutes found are shown in Figure 2. The pooled fractions were concentrated by rotary evaporator and two component fractions namely PF1 and PF2 were obtained. The PF1 fraction was found only to have antimicrobial activity against selected microbes when tested with agar well diffusion method.

The PF1 was then subjected to LC-MS (6410 Triple Quadrupole LC/MS, Agilent Technologies, USA); FT-IR (Nicolet iS10, Thermo Scientific, USA) and Nuclear Magnetic Resonance (NMR) (Make: Varian, Model: Mercury plus**).**

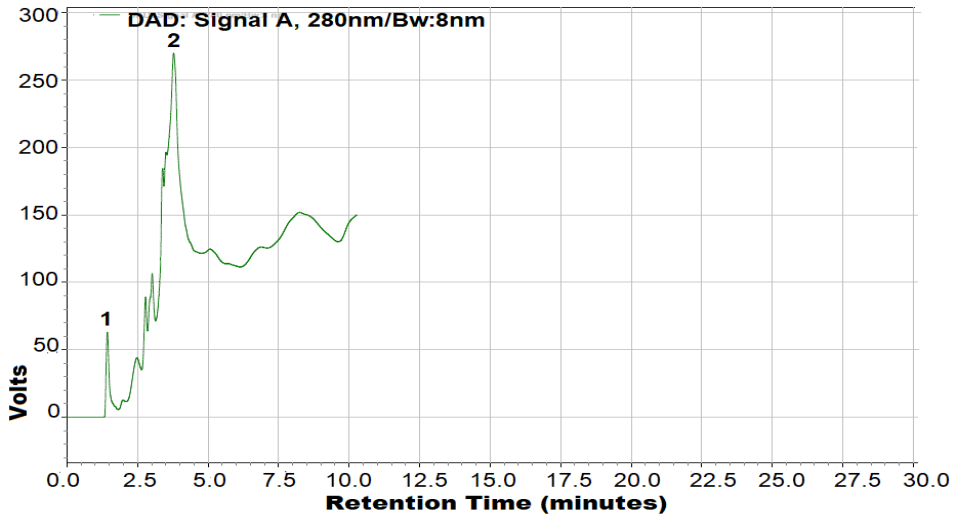


Figure 2: Preparative HPLC profile, the numbers showed purified fractions 1 – 2 were collected

Preparation of herbal gel

The composition of herbal gel formulation is given in Table 1. The herbal hydrogel was formulated by dissolving PF1 (1% w/w) in water followed by addition of PG (5% w/w) and sodium benzoate (0.25% w/w) into it in a mortar with continuous trituration with a pestle. To this aqueous mixture, C934 (2% w/w) was slowly dispersed with tituration. The pH of the hydrogel was adjusted with addition of TEA dropwise with titration until desired pH 6.8–7.0 was achieved[41]. The prepared hydrogel was packed in wide mouth jar covered with a lid and was kept in dark at room temperature for 24 hours to equilibrate the herbal hydrogel before performing rheological measurement[42]. The blank hydrogel was prepared using the same method without addition of herbal extract.

Table 1: Composition of herbal gel

Material	**%(w/w)**
Carbapol 934 (C934)	2
Propylene glycol (PG)	5
Herbal extract (PF1)	1
Sodium benzoate	0.25
Purified water	q.s. to 100
Triethanolamine (TEA)	q. s.

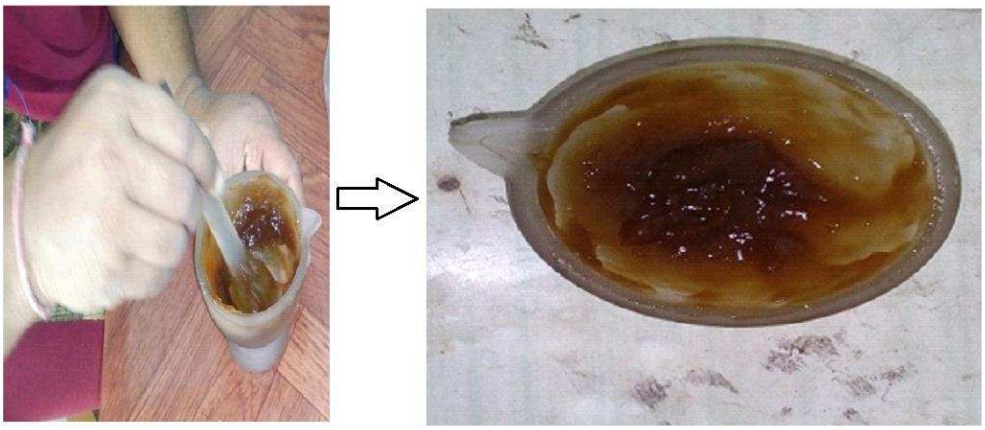


Figure 3: Prepared Herbal Gel

Evaluation of herbal gel

Visual Examination: The physical properties of the prepared herbal hydrogel (Figure 3) were compared with blank hydrogel formulation (without the herbal extract), Renicol and Daktarin. Gels were inspected visually for their color, homogeneity (appearance and presence of any aggregates), grittiness (presence of particles or grits), and syneresis (phase separation).

Determination of pH: The pH of hydrogel formulations were determined using digital pocket pH meter (Mettler Toledo, Switzerland), which was calibrated before each use with standard buffer solutions. A quantity of 1 g of gel was dissolved in 100 mL of distilled water and stored for two hours. The electrode was inserted into the solution 10 min prior to recording the reading at room temperature. The measurements were carried out in triplicate and average pH was calculated[43].

Spreadability Test: The spreadability of the prepared and marketed gel formulations were determined by putting 1.0 g of gel on a glass plate (10 cm × 15 cm) and then covering the gel with a second glass plate over it and finally pressing the plates by putting 500 g of weight on them for 5 minutes. The diameter of the circle created by gel after spreading was measured with a centimeter scale. A higher value of the diameter of the gel circle means more easily the gel can be spread[44].

Determination of Viscosity: The viscosity of the prepared herbal hydrogel and marketed gels were determined using a Rheometer (Model: R/S Portable Rheometer, Make: Brookfield Viscometers Ltd., UK) with spindle #C50 at a speed of 50 rpm. All measurements were done in triplicate at room temperature[45].

Stability Study: The prepared hydrogel was stored in airtight glass containers at room temperature (25°C) and cold temperature (4-8°C) over a period of three months (Chowdhary and Kumar 1996). After three months, samples were visually observed for a change of color, odor and phase separation. Any change in pH of the formulations was also determined[46].

***In vitro* Antimicrobial assay of herbal gel**

The *in vitro* antimicrobial activity of the prepared herbal gel was determined using agar well diffusion method against *Staphylococcus aureus* for antibacterial activity in Mueller-Hinton Agar and *Candida albicans* for antifungal activity in Sabouraud-Dextrose Agar.

Antibacterial assay: 15 mL of Mueller-Hinton Agar was poured in sterilized petri dishes and allowed to air dry under laminar air flow (Macro Scientific Works, CAT No: MSW-162). After solidification of agar, plates were inoculated with 100 µL of overnight grown bacterial culture adjusted to 0.5 McFarland Turbidity Standards. After inoculation, wells were made using sterile cork borer and one-third of the well was filled with prepared hydrogel. Plates were incubated for 24 hours in a BOD incubator (ORBITREK. BODY.350, Scigenics Biotech Pvt. Ltd, Chennai, India) at 35° C (Relative humidity ≥ 80%) and zone of inhibition was measured after 24 hours (Figure 4). Here, blank gel and Renicol were taken as negative and positive controls, respectively[39].

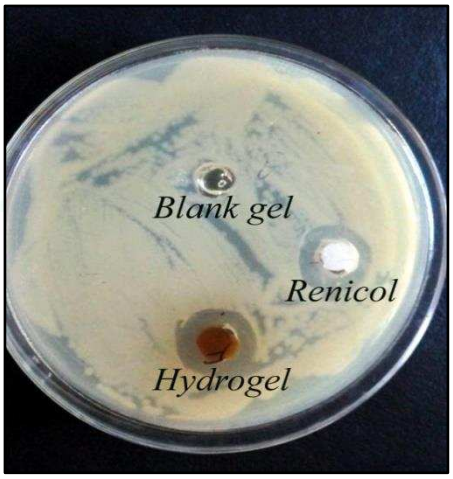


Figure 4: Antibacterial activity evaluation of Hydrogel, Renicol and Blank gel against *Staphylococcus aureus*. The hydrogel prepared from PF1 of methanolic leaf extract of *Cassia alata* shows a better antibacterial activity as compared to the blank and marketed gel formulation.

Antifungal assay: 15 ml of Sabouraud-Dextrose Agar media was poured on glass plates and allowed to air dry under the laminar air flow. After solidification, plates were inoculated with 100 µL of fungal strains and wells were made by using sterile cork-borer. Then one-third of the well was filled with formulation and plates were kept in BOD incubator for 48 hours at 28° C (Relative humidity ≥ 80%) and the zone of inhibition was measured at 48 hours (Figure 5). Here blank gel was taken as negative control, whereas Daktarin was taken as positive controls[39].

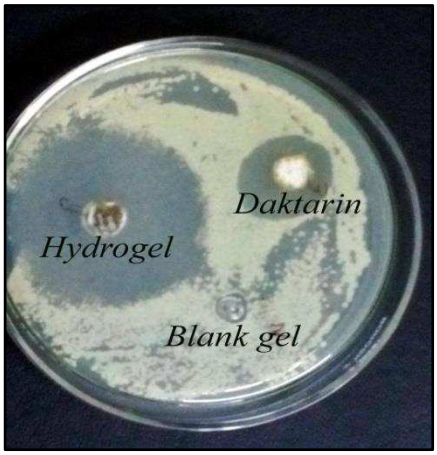


Figure 5: Antifungal activity evaluation of Herbal gel, Renicol and Blank gel against *Candida albicans*. The hydrogel prepared from PF1 of the methanolic leaf extract of *Cassia alata* shows a better antifungal activity as compared to the blank and marketed gel formulation.

Determination of Minimum Inhibitory Concentration (MIC) against microbes: The MIC of the prepared herbal gel against bacterial and fungal strains was tested by broth dilution method. The prepared gel was dissolved in distilled water to obtain 1000 µg/mL stock solution. For the evaluation of antibacterial activity, 0.5 mL of the stock solution was incorporated with 0.5 mL of Mueller-Hinton Agar broth to make 1000 µg/mL solution. From this solution, different concentrations were made in serial dilution at 200µg/mL, 180µg/mL, 160µg/mL, 140µg/mL, 120µg/mL, 100µg/mL, 80µg/mL and 60µg/mL Here the negative control was the test organism and the positive control was test organism with Renicol. The culture tubes were incubated in a BOD incubator at 35°C for 24 hours (Relative humidity ≥ 80%). The lowest concentration which did not allow any visible growth of tested organism was taken as MIC[47].

For the evaluation of antifungal activity, 0.5 mL of the stock solution was incorporated with 0.5 mL of Sabouraud-Dextrose Agar broth to make 1000 µg/mL solution. From this solution, different concentrations were made in serial dilution at 200 µg/mL, 180 µg/mL, 160 µg/mL, 140 µg/mL, 120 µg/mL, 100 µg/mL, 80 µg/mL and 60 µg/mL. The negative control tube was containing broth with test organism and the positive control was test organism with Daktarin. The culture tubes were incubated in a BOD incubator at 28°C for 48 hours. The lowest concentration which did not allow any visible growth of tested organism was taken as MIC.

The *in vivo* antimicrobial efficacy of herbal gel

The antimicrobial and wound healing efficacy of the developed hydrogel were investigated in rat model with surgical site infection. Healthy Wister albino rats (150-200 g) of both sexes were used for the study and a total of 18 rats were used in the study. Animals were purchased from M/S. Saha Enterprise, Kolkata, West Bengal. The Institutional Animal Ethical Committee (IAEC), of Dibrugarh University, Dibrugarh, Assam, India, approved (Approval No: IAEC/DU/139 Dated-29.10.2016) the experimental protocol. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) to the Care and Use of Experimental Animals. The animals were kept in polypropylene cages (55×32.7×19 cm, bottom covered with sawdust litter) in a temperature controlled environment (25 ± 1ºC). Lighting was controlled to supply 12 hours of light and 12 hours of dark for each 24 hours period. Animals were acclimated with free access to drinking water and standard diet for 10 days before the experiment.

To infect the surgical wounds of rats *Staphylococcus aureus* (bacterial strain) and *Candida albicans* (fungal strain) was used[30]. The bacterial inoculum was prepared in Mueller-Hinton Agar broth and fungal inoculum was prepared in Sabouraud-Dextrose Agar broth. The bacterial and fungal inoculums were adjusted to 0.5 McFarland Turbidity (1.5×10^8 CFU/ml) (CFU= Colony forming units) Standards.

Rats were divided into 6 groups each containing 3 rats. Rats of each group were kept in separate cages. Three groups were infected with the bacterial strain and another three groups were infected with fungal strain. For the *Staphylococcus aureus* infected set, the groups were Control group (challenged with the bacteria and did not receive any antibiotic); Standard group (challenged with the bacteria and treated with Renicol) and Test group (challenged with the bacteria and treated with prepared herbal gel). Similarly, the *Candida albicans* infected groups were Control group (challenged with the fungus and did not receive any antifungal drug), Standard group (challenged with fungus and treated with Daktarin) and Test group (Challenged with the fungus and treated with prepared herbal gel). One hour before creating the infections, animals were anesthetized with intraperitoneal Urethane (i. p., 1.2 g/kg b. w.). When the rats became unconscious, hair of the lower back area of the rat was shaved and the skin was cleansed with 70 %v/v Ethanol. A 1-cm-long, full thickness incision wound was created in the shaved and cleaned area. Approximately, 1cm of absorbable silk suture infected with *Staphylococcus aureus* for the bacteria infected animal groups and *Candida albicans* for fungus infected set (approximately 5×10^3 cells/cm of suture) was placed into the wound and secured in the skin by knotting. One single suture was attached to the middle of the incision. Then the animals were returned to the individual cages[44]. The process of creating infections has been presented in Figure 6.

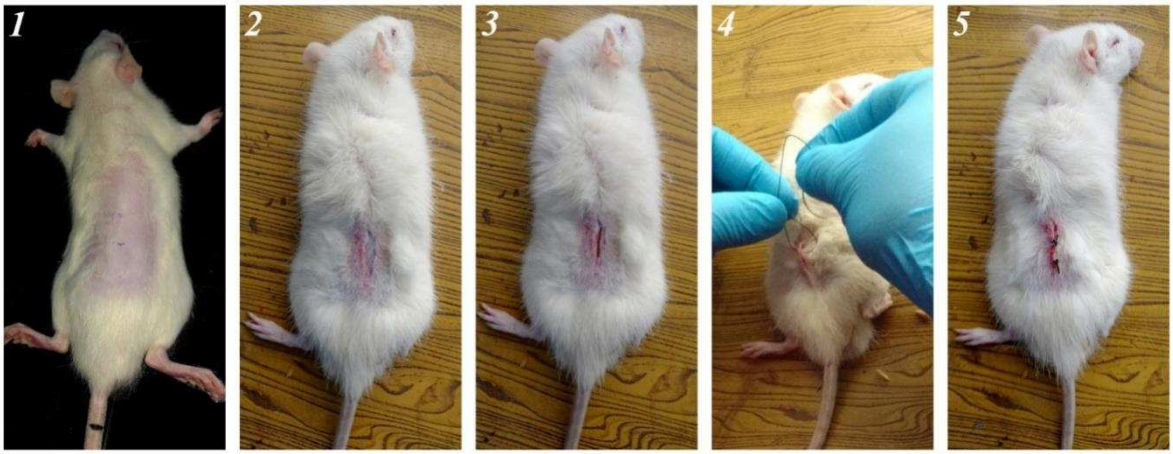


Figure 6: Creating infection with Bacterial and fungal strains in rats. (1) Shaving and cleaning the lower back area of Rat; (2) Making 1cm long full thickness incision on the cleaned area; (3) Placing 1cm long absorbable silk suture dipped in *Staphylococcus aureus*/ *Candida albicans* suspension; (4) Stitching the cut area to secure the suture; (5) The infected wound of the rat.

Treatment with antimicrobial formulations was started 24 hours after incision and suturing. The treatment was continued for 3 weeks. Measurement of the wound and pictures of healing process were taken every day. After 3 weeks of treatment, the animals were sacrificed by cervical dislocation method. 1 cm^2 tissue was cut from the infected area to examine the effectiveness of the prepared gel by the bioburden study. Equal sections of the isolated skin tissues were used for all the animal groups to read the bacterial and fungal bioburden. The viable counts of bacteria and fungus per section were analysed in the tissue homogenate. Quantification of the viable bacteria and fungus in the homogenate was done by culturing serial 10-fold dilutions of the bacterial suspension onto Mueller-Hinton agar plates and fungal suspension on Sabouraud-Dextrose agar plates. The plates were incubated at 37^oC for 24 hours for bacterial strains and 48 hours for fungal strains. The organisms were quantified by counting the number of CFU/section using a colony counter (LA660, HIMEDIA, India).

Statistical Evaluation

Statistical analysis was performed using Graph Pad Prism version 7 (USA). All experiments were conducted in triplicate and results were expressed as the mean±SD. Data from each experiment were analysed statistically by one-way ANOVA. Multiple comparisons among groups were determined with Tukey test. Differences were considered significant when the p-value was less than 0.05 ($p<0.05$).

RESULTS AND DISCUSSION

The methanolic leaf extract of *Cassia alata* L. was tested for antimicrobial activity against bacterial strains viz. *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli* and fungal strains viz. *Candida albicans, Microsporum Canis,* and *Trichophyton rubrum* using agar well diffusion method and showed antimicrobial activity against the selected microorganisms. The zones of inhibition of methanolic leaf extract of *Cassia alata* L. are presented in the Table 2.

This extract produced one highly bioactive fraction upon silica gel column chromatography, which showed significant antimicrobial activity against tested bacterial (*Staphylococcus aureus, Staphylococcus epidermidis)* and fungal (*Candida albicans*, *Microsporum canis*) strains. The zones of inhibition of this bioactive fraction against selected microbes are given in the Table 3. TLC of this bioactive column fraction produced two spots on TLC plate indicating the presence of more than one component in the fraction (Figure 7). So it was further purified with Preparative HPLC, which produced two pure fractions, namely PF1 and PF2, and only PF1 found to be bioactive as it showed antimicrobial activity against test organisms. The zones of inhibition of PF1 against selected microbes are given in the Table 4. As PF1 was found to be bioactive, it was further analysed with FT-IR, LC-MS and NMR.

The spectral figures of FT-IR, LC-MS and NMR are given in Figure 8, Figure 9 and Figure 10, respectively.

Table 2: Comparison of antimicrobial activity of methanolic leaf extract of *Cassia alata.* L. with standard drug against selected microbes

Microbes	Diameter of Zone of Inhibition (mm)#		
	Methanolic leaf extract of *C. alata* L.	Standard antibacterial drug (Chloramphenicol)	Standard antifungal drug (Nystatin)
Staphylococcus aureus	24.66 ± 0.33	22.00 ± 0.33	nd*
Staphylococcus epidermidis	22.66 ± 0.33	21.00 ± 0.33	nd*
Escherichia coli	21.34 ± 0.33	20.00 ± 0.33	nd*
Candida albicans	23.66 ± 0.33	nd*	22.00 ± 0.33
Microsporum canis	21.66 ± 0.33	nd*	21.00 ± 0.33
Trichophyton rubrum	21.33 ± 0.33	nd*	20.00 ± 0.33

Mean ± SD (n = 3); *nd = not determined.

Table 3: Comparison of antimicrobial activity of bioactive fraction of methanolic leaf extract of *Cassia alata* L. with standard drug against selected microbes

Microbes	Diameter of Zone of Inhibition (mm)¥		
	Bioactive fraction from column chromatography	Standard antibacterial drug (Chloramphenicol)	Standard antifungal drug (Nystatin)
Staphylococcus aureus	28.67 ± 0.33	22.00 ± 0.33	nd**
Staphylococcus epidermidis	25.67 ± 0.67	21.00 ± 0.33	nd**
Candida albicans	21.67 ± 0.67	Nd**	21.33 ± 0.89
Microsporum canis	19.67 ± 0.67	nd**	20.00 ± 0.33

¥ Mean ± SD (n = 3); ** nd = not determined

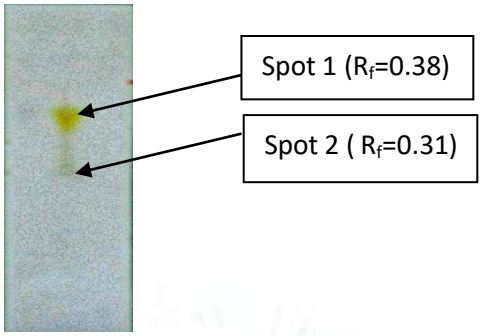
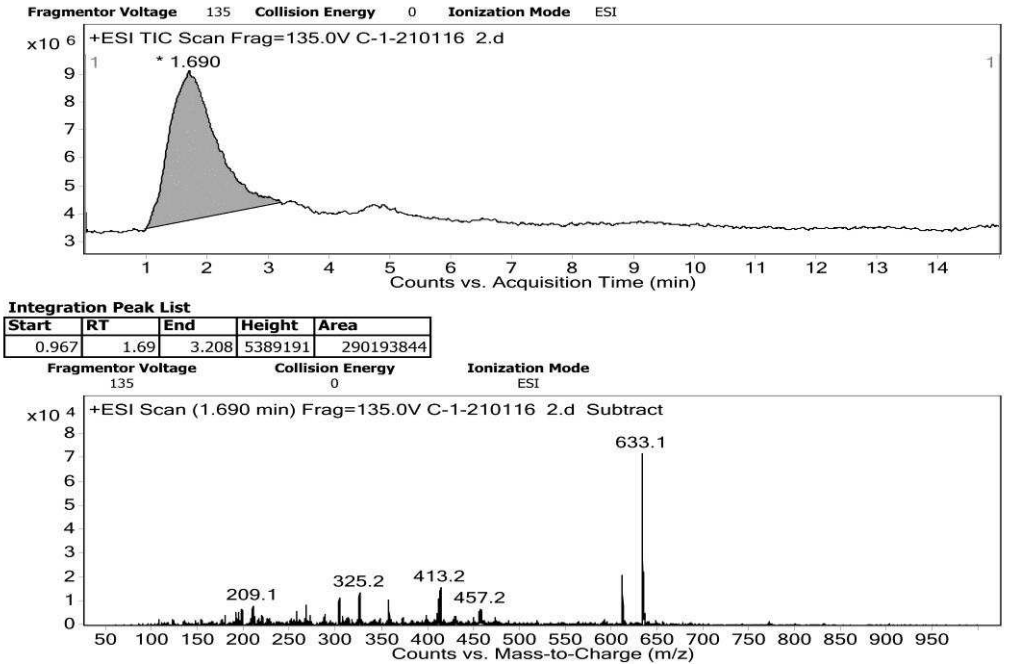


Figure 7: TLC of bioactive fraction (binary fraction of ethyl acetate and ethanol) of methanolic leaf extract of *Cassia alata* L.

Table 4: Evaluation of antimicrobial activity of PF1 and its comparison with standard antimicrobial drugs against selected microbes

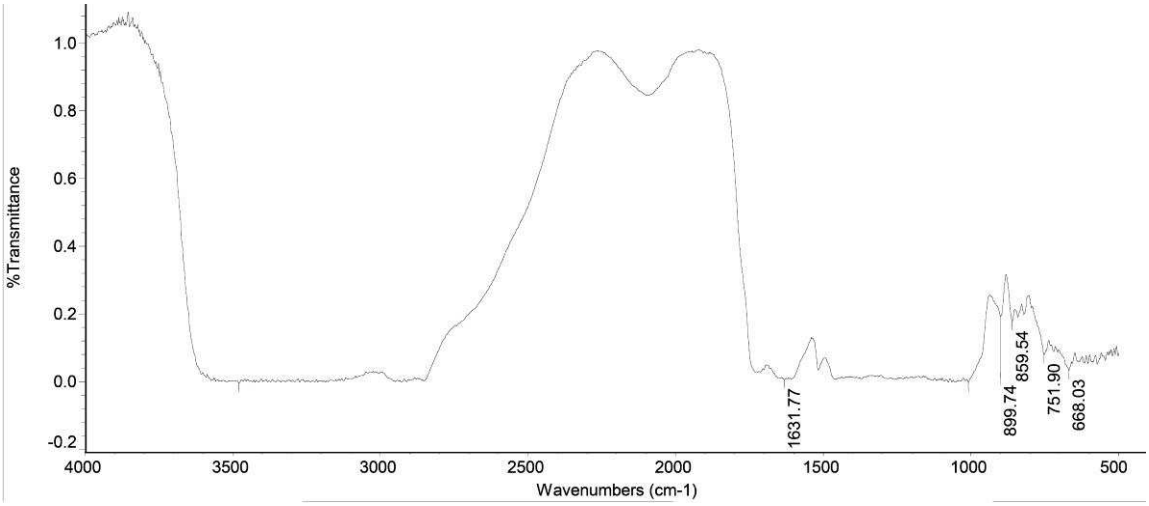
Microbes	Diameter of Zone of Inhibition (mm)θ		
	PF1	Standard antibacterial drug (Chloramphenicol)	Standard antifungal drug (Nystatin)
Staphylococcus aureus	14.66 ± 0.33	20.00 ± 0.33	nd***
Escherichia coli	12.34 ± 0.67	19.64 ± 0.33	nd***
Candida albicans	10.33 ± 0.33	nd**	14.64 ± 0.33

θMean ± SD (n = 3); ***nd= not determined



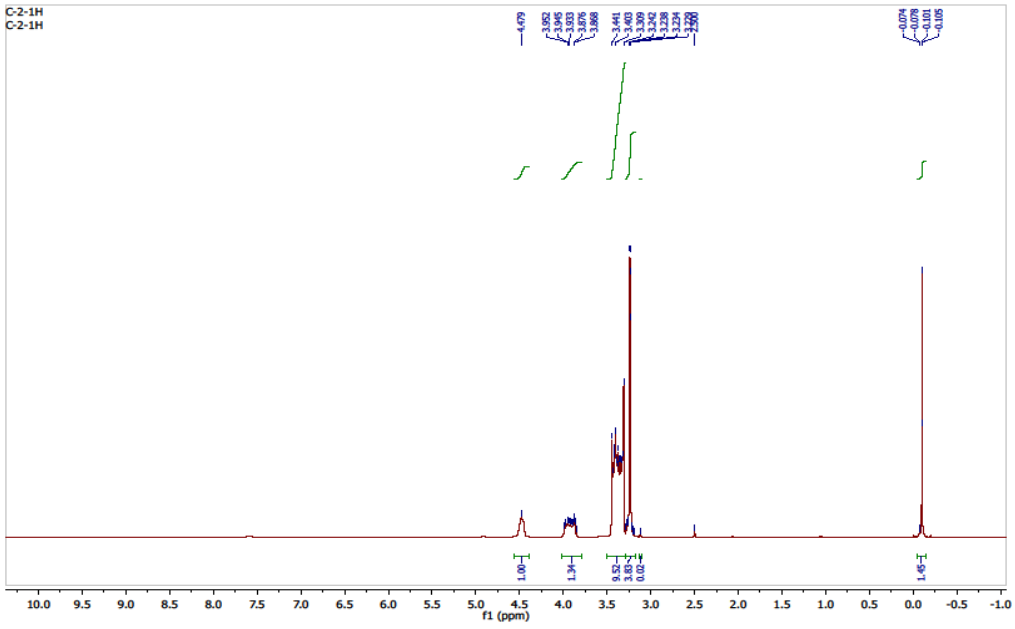
ESI-MS m/z: 633.1

Figure 8: LC-MS analysis of PF1



FT-IR (KBr): 3500 (OH), 1750 (C=O) cm^{-1}.

Figure 9: FT-IR analysis of PF1



^{1}H-NMR (400MHz, DMSO-d_6): d 4.47 (bars, 1H), 4.03 (bars, 1H), 3.58-3.38 (m, 8H), 3.36-3.17 (m, 3H**).**

Figure 10: ^{1}H-NMR NMR analysis of PF1

From the FT-IR spectral data of the PF1 bioactive fraction, it can be concluded that PF1 may contain alcohol and ketone functional group. It was revealed from the ^{1}H-NMR analysis that the compounds are aliphatic in nature, since no peaks were observed around 6-8 ppm. In ^{1}H-NMR spectra of the compound, the peak was in the range of 3-4 ppm. So this may be due to the presence of heteroatom in the compound. The antimicrobial activity of PF1 may be due to the presence of alcohol and ketone group in the compound. This pure bioactive fraction was used to prepare the herbal antimicrobial gel.

Physical evaluation of herbal gel

The prepared hydrogel was glossy brownish-green in color, homogeneous, with the absence of any lump or grittiness and syneresis. The pH value of the prepared herbal hydrogel was 7.0. It is considered acceptable to avoid skin irritation upon application[48]. Good spreadability is one of the criteria for the gel to meet ideal qualities. It is the term expressed to denote the extent of the area to which gel readily spreads on the application. Therapeutic efficacy of a gel formulation also depends on its spreading value[42]. Additionally, spreadability is very important as it shows the behavior of the gel when it comes out from the tube[29]. The tabulated spreadability values indicate that the polymers used in the hydrogel formulation have spreading capacity with the shearing force of low magnitude. It can be concluded that the prepared hydrogel fulfilled the requirement of gel-based formulations for dermatological application which should have several favorable properties such as greaseless and ease of spreadability[19,27]. The viscosity of gel formulation is an important physical property of topical formulations, which affects the rate of drug release[45]. The viscosity of the prepared hydro gel was found to be at 15.73 Pa-s. The result is shown in Table 5.

Table 5: Comparison of physical properties of hydrogel, blank gel, marketed antibacterial gel and antifungal gel

Physical properties	Blank gel	Hydrogel	Renicol	Daktarin
Appearance	Thick Gel	Thick Gel	Ointment	Less viscous gel
Grittiness	Non-gritty	Non-gritty	Non-gritty	Non-gritty
Color	Transparent	Brownish Green	Translucent	White
pH	7.0±0.1	7.0±0.1	6.8±0.2	7.1±2.0
Spreadability (cm.)	5.93±0.10	6.43±0.22	4.35±0.17	7.73±0.13
Stability	Stable	Stable	Not studied	Not studied
Viscosity (Pa-s)	15.58	15.73	9.52	2.88

Values are expressed as the mean ± SD (n=3)

Stability of herbal gel: The prepared gel showed promising stability at the refrigerator and ambient room conditions for three months. The prepared herbal gel did not show any change in color, odor, pH, drug content, and rheological properties. Additionally, no phase separation was observed. This indicated that the PF1 was stable in gels even after three months of short-term storage and the gel formulation was physically and chemically stable[49]. It is clear from the above discussion that prepared hydrogel formulation meets all the parameters of a standard gel. Therefore, it was subjected to further investigation for microbiological activities[50-52].

***In vitro* Antimicrobial assay of herbal gel:** The agar well diffusion method was used for screening the antimicrobial potential of prepared herbal gel formulation and the results are shown in Table 6. The herbal gel gave a larger zone of inhibition than the marketed formulations against the test organisms. This difference between the zone of inhibitions of herbal gel and marketed antimicrobial formulations is statistically significant ($p<0.05$). Further, the MIC value of herbal gel is lesser than the marketed antifungal and antibacterial formulations (Table 7). The results of *in vitro* antibacterial assay clearly indicate the higher antimicrobial efficacy of herbal gel than that with marketed antimicrobial formulations.

Table 6: Comparison of *in vitro* antibacterial and antifungal activity of herbal gel and marketed antimicrobial formulations against *Staphylococcus aureus* and *Candida albicans*

Microbes	Zone of inhibition (mm)ø		
	Herbal gel	Renicol	Daktarin
Staphylococcus aureus	20.33 ± 0.33[a]	19.00 ± 0.33	Not determined
Candida albicans	31.66 ± 0.65[b]	Not determined	25.45 ± 0.65

øMean ± SD (n = 3). Note: Data were significantly different ($p < .05$), when zone of inhibition with herbal gel against *Staphylococcus aureus* and *Candida albicans* were compared for zone of inhibition with standard antibacterial Renicol and antifungal Daktarin, respectively, by one-way analysis of variance (ANOVA) through Tukey's multiple comparisons test. [a]Data were significantly different ($p = 0.0065$), where herbal gel and renicol were compared with blank gel(with no zone of inhibition) by one-way ANOVA through Tukey's multiple comparisons test. [b]Data were significantly different ($p = <0.0001$), where herbal gel and daktarin were compared with blank gel(with no zone of inhibition) by one-way ANOVA through Tukey's multiple comparisons test..

Table 7: Comparison of MIC of herbal gel and marketed antimicrobial formulation against *Staphylococcus aureus* and *Candida albicans*

Microbes	MIC (µg/ml)		
	Herbal gel	Renicol	Daktarin
Staphylococcus aureus	80	100	Not determined
Candida albicans	120	Not determined	160

***In vivo* antimicrobial efficacy of herbal gel**:

Visual examination of healing of wound infected with bacterial and fungal strains in rat groups shows that herbal gel treated group possesses better and fast healing properties than control as well as marketed antimicrobial formulation treated group. The herbal gel is better in healing the bacterial and fungal infected wounds as compared to the marketed formulations. The contraction of wound length in bacterial and fungal infected rats treated with herbal gel is significant higher than those treated with marketed formulation ($p<0.05$). The detailed results of the wound length have been given in Table 8. The wound healing process of the rats infected with bacteria after 1st day, 10 days and 21 days of study have been given in Figure 11 (Test group), Figure 12 (Control group) and Figure 13 (Standard group). The wound healing process of the rats infected with fungus after 1st day, 10 days and 21 days of study have been given in Figure 14 (Test group), Figure 15 (Control group) and Figure 16 (Standard group).

Table 8: Contraction of wound length with application of herbal gel and marketed antimicrobial formulations

Microbial strain(s)	Animal groups	Length of wound (cm)			
		Day 1	Day 7	Day 14	Day 21
Staphylococcus aureus infected set	Control	1	0.7 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
	Standard	1	0.6 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
	Test	1	0.5 ±0.1[c]	0.1 ±0.1[d]	0.00[e]
Candida albicans infected set	Control	1	0.8 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
	Standard	1	0.9 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
	Test	1	0.6 ± 0.1[f]	0.2 ±0.1[g]	0.00[h]

Note: Data were expressed as mean±SD (n = 3). Data were significantly different ($p < .05$), where wound lengths at day 7, 14 and 21 were compared for control, standard and test animal group by one-way analysis of variance (ANOVA) through Tukey's multiple comparisons test. [c]Data were non –significant ($p = 0.2694$), where test and standard were compared with control by one-way ANOVA through Tukey's multiple comparison test. [d]Data were significantly different ($p = 0.0003$), where test and standard were compared with control by one-way ANOVA through Tukey's multiple comparison test. [e]Data were significantly different ($p = 0.0003$), where test and standard were compared with control by one-way ANOVA through Tukey's multiple comparison test. [f]Data were significantly different ($p = 0.0006$), where test and standard were compared with control through Tukey's multiple comparison test. [g]Data were significantly different ($p = <0.0001$), where test and standard were compared with control by one-way ANOVA through Tukey's multiple comparison test. [h]Data were significantly different ($p = <0.0001$), where test and standard were compared with control by one-way ANOVA through Tukey's multiple comparison test.

The bioburden study results revealed that tissue homogenate from herbal gel treated rat group contained less number of bacterial and fungal cell than control as well as marketed formulation treated groups (Table 9). The bioburden in rats treated with herbal gel is significantly lower than those treated with marketed formulation ($p<0.05$). This result shows that developed herbal gel containing PF1 of leaf extract of *Cassia alata* L. not only heals the wound faster than the marketed formulation, but also it reduces the number of infection causing bacteria and fungus. In this way, the prepared herbal gel formulation heals the wound by killing the wound causative organisms. Hence, it is more efficient in healing the wounds than the marketed antimicrobial formulations.

Table 9: Total plate count of bacterial and fungal cell present in the tissue homogenate

Rat set	Total plate count in CFU/ml		
	Control group (I)	Standard group (I)	Test group (I)
Staphylococcus aureus infected set	297 ± 0.33	215 ± 0.57	67 ± 0.33[i]
	Control group (II)	Standard group (II)	Test group (II)
Candida albicans infected group	269 ± 0.57	204 ± 0.57	127 ± 0.57[j]

Note: Data were expressed as mean±SD (n = 3). Data were significantly different ($p < .05$), where total plate count for control, standard and test group were compared by one-way analysis of variance (ANOVA) through Tukey's multiple comparisons test. [i]Data were significantly different ($p = <0.0001$), where test group and standard group were compared with control group by one-way ANOVA through Tukey's multiple compariosons test. [j]Data were significantly different ($p = <0.0001$), where test group and standard group were compared with control group by one-way ANOVA through Tukey's multiple compariosons test.

Figure 11: Bacteria infected set treated with herbal gel

Figure 12: Bacteria infected set (control group)

Figure 13: Bacteria infected set treated with the Standard formulation

Figure 14: Fungus infected set treated with herbal gel

Figure 15: Fungus infected set (control group)

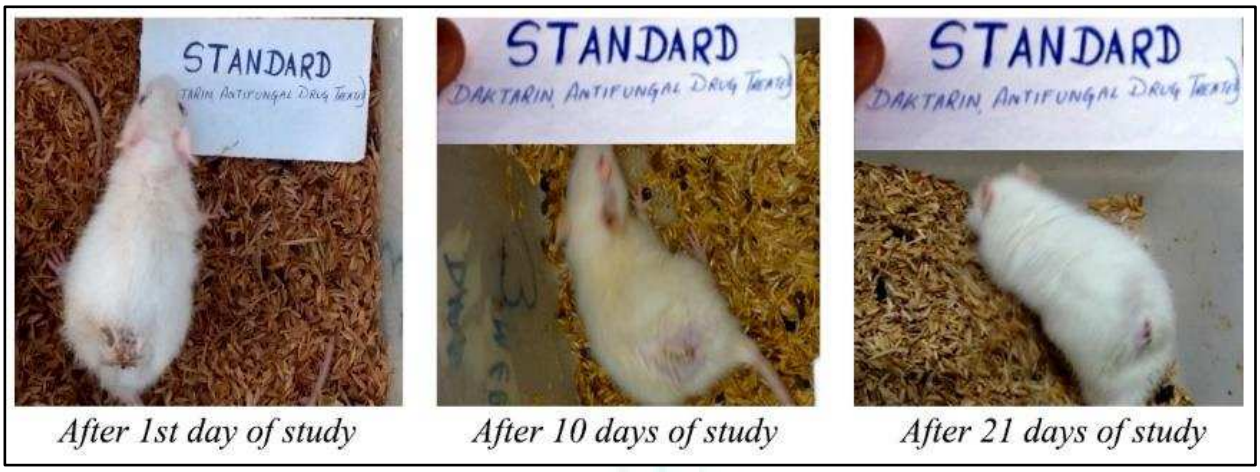


Figure 16: Fungus infected set treated with the Standard formulation

CONCLUSION

Methanolic leaf extract of *Cassia alata* (L.) was successfully purified to a bioactive fraction namely PF1. This leaf extract exhibited significant antibacterial and antifungal activity against the tested bacteria (*Staphylococcus aureus* MTCC 9542) and fungi (*Candida albicans* MTCC 4842). The novel herbal hydrogel containing a pure bioactive fraction of *Cassia alata* (L.) was successfully developed and evaluated as a potent antimicrobial gel both *in vitro* and *in vivo* compared to the marketed antimicrobial formulations. The bioburden of microbes in the skin tissue homogenate of rats treated with hydrogel and marketed formulation indicated that the prepared herbal gel had the better killing of microbes in the skin tissue than the marketed formulation. This herbal gel formulation could be very promising and innovative topical alternative for treatment of skin infections caused by bacteria as well as fungal strains without hazard to human health based on the fact of its traditional use by the Assamese people with no toxic effects. These findings may open new avenues for the treatment of dermal infections by local application of the herbal antimicrobial gel. However, further preclinical and clinical studies are recommended to support its efficiency claims in humans. Further approaches are needed to clearly elucidate the full mechanism of action of such natural preparations in the healing of wound.

Conflict of Interest: None

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