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Full Length Research Paper

Design, development, and evaluation of antimicrobial activity of herbal antiseptic wound pad-neemplast

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There is growing demand of polyherbal formulations in the world market. The present study aimed to design, develop, and evaluate the antiseptic activity of herbal wound pad Neemplast containing distillate of Neem leaves *(Azadirachta indica)* and Black pepper *(Piper nigrum)* and the oil of clove *(Syzygium aromaticum)* and Eucalyptus (*Eucalyptus globules)*. The plants have been reported in the literature as having good antimicrobial, anti-oxidant and anti-inflammatory activity. Various formulation batches were prepared and evaluated for various parameters like colour, appearance, pH, weight per ml, assay identification and antimicrobial activity. The formulation of Batch# 358-K-12 was compared with the marketed preparation Sani-plast. It is a very good attempt to establish the herbal antiseptic wound pad containing distillate of Neem leaves Black pepper. The plant trials were conducted on a commercial scale machines in order to observe behavior and feasibility of machine with respect to new product formulation and also initiated analytical studies (qualitative and microbiological determination) in order to get the physical and chemical compatibility of formulation with the wound pad material. Implement current good manufacturing practice (cGMP) concept during all the manufacturing and packaging process. Neemplast wound pad was successfully designed and developed after extensive manufacturing and evaluation process by specialized techniques for evaluation of antiseptic activity *in vitro*.

Key words: Antiseptic, distillate, *Piper nigrum, Azadirachta indica.*

INTRODUCTION

Neemplast wound pad consist of sterilized non-woven (70% viscose 30% polyester) antiseptic pad impregnated with Neem leaves *(Azadirachta indica)* and Black pepper *(Piper nigrum)* distillate along with Clove *(Syzygium aromaticum)* and Eucalyptus oil (*Eucalyptus globules)* which is covered by releasing and printed paper (Wink and Wyk, 2004; PDR, 2004). It is used to protect and heal cuts, scratches, blisters, insect bites and minor wounds. It is new antiseptic polyester fabric adhesive bandages as the elementary material is more suitable to be used for children as well as for adults. For its more advantages, each bandage is wrapped individually in a waterproof bag. The outer package is strong cardboard carton to keep best storing condition (Monteiro-Riviere et al., 2005). Neem leaves show pharmacological actions as, analgesic, antibacterial, antifungal, and anti-inflammatory properties (Parotta, 2001; Ross, 2001). Clove oil possesses antimicrobial potential (Dorman and Deans, 2000; Betoni et al., 2006). Eucalyptus oil has anti-inflammatory, analgesic and antimicrobial activities used

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Table 1. Neemplast specifications for bulk solution and finished product (strip).

Parameter	Norm
Appearance	Light yellow to colorless transparent liquid with characteristic odor
pH	4.5 – 6.5
Weight per ml	(0.967 – 1.070) g/ml
Assay Identification: Eugenol	Positive
Finished product (bandages)	
Description	Transparent P.E film ventilated bandage pad impregnated with Neem and black pepper distillate along with clove and eucalyptus oil which is covered by releasing paper and printed paper
Identification	Eugenol
Adhesive skin test	Easily removes from skin without any adhesive deposition.
Average weight (g)	0.5226±5% (0.494 –0.546)
Bandage length (mm)	72±1
Bandage width (mm)	19±1

traditionally (Cimanga et al., 2000; Benayache et al., 2001; Boland and Brophy, 1991). Black piper (*Piper nigrum*) has antimicrobial activity and is used as antibacterial against different microorganisms (Dorman and Deans, 2000; Perez and Nesini, 1994). Its designed antiseptic polyester fabric adhesive bandages as the elementary material is more suitable to be used for children as well as for adults. Each bandage is wrapped individually in a waterproof bag. The outer package is strong cardboard carton to keep best storing condition. The idea for designing the Neemplast was taken from the Shanghai Huazhou PSA Products Co., Ltd. This prospective study was to be conducted to define and prove that the Neemplast purported function in a consistent and reproducible manner as per manufacturing process. Neemplast is manufactured by combination of medicinal plants and there is no synthetic material used in manufacturing of Neemplast wound pad. This type of innovation is alternate to synthetic wound pad Sani-plast with almost similar efficacy and antiseptic activity. *A. indica* and *P. nigrum* are reported to possess very beneficial effect on skin due to their anti-microbial, anti-inflammatory and anti-oxidant activities.

MATERIALS AND METHODS

Collection of plant material

Leaves of neem and black pepper were purchased from the local market Jooria bazaar, Karachi while clove oil and eucalyptus oil were collected from the Supply Chain Department of Herbion Pakistan Private Limited.

Preparation of extracts

1.25 kg of dry Neem leaves was taken and properly washed. 8.75 liters of D.I water in extractor was collected and herb placed in it. This was stirred and heating till boiling. The temperature was from 110-120°C. Then temperature was reduced and maintained up to 90-100°C for 30 min. When extraction was completed, the steam was release and the aqueous extract filtered through mesh number 100. After filtration, the filtrate was transferred to evaporator. The filtrate was concentrated through evaporation. The temperature was from 100-110°C. 1.25 gm of methyl paraben and 0.25 gm of propyl paraben was added to the concentrated thick extract and stirred for 10 min, and the desired concentration of extract was obtained.

Development of formulations

Different batches were prepared according to the Table 1. The desired concentration of extract, oils and emulsifying agent were weighed accurately and following these parameters light viscous and smooth texture that gave shiny, soft surface with smooth impregnation, low squeezer gauge 4.4 mm for more retention of active on non impregnated wound roll and kept low dryer temperature 170 -172°C that role in prevention from over drying and degradation of actives and reduced stress and load on dryer heater, high speed F 40.45 Hz for maximum output of product. Prepared the solution and finally dipped the wound pad roll into the bulk solution. The specification of bulk and finished product is shown in Table 1. The manufacturing of herbal antiseptic neem plast wound pad involves the following stages: Preparation of neemplast solution and impregnation of wound pad

Preparation of Neemplast solution

First step in manufacturing of Neemplast is preparation of Neemplast solution which involves preparation of two phases separately and then mixing them together to get the final solution.

Phase I (water phase): Took accurate amount or quantity of Neem and Black pepper distillate previously obtained from Neem leaves (that is, 500 gm) and black pepper fruit (12.5 gm) in stainless steel vessels. Then add Benzalkonium chloride and mixed well with constant stirring for 10 min. Added and dissolved EDTA to got clear, transparent solution.

Phase II (oil phase): Took Eucalyptus and clove oil in a beaker and mixed well. Added hydrogenated castor oil and polysorbate 20 and mixed with continuous stirring. Then transferred gently Phase II (Oil phase) into Phase I (water phase) with constant stirring to got homogenous uniform and clear solution. Make up volume with distilled water and mixed well. Then checked the pH of solution that was in range of 5.0- 6.0.

Table 2. Parameters for neemplast assessment.

S/N	Parameter	Norm
1	Description	Transparent P.E film ventilated bandage pad impregnated with Neem, Black pepper distillate along with Clove and Eucalyptus oil which is covered by releasing and printed paper.
2	Identification	Eugenol (must be positive)
3	Adhesive skin test	Easily removes from the skin without any adhesive deposition.
4	Average weight of strip with releasing and packing paper (g)	0.5226±5% (0.495 – 0.546)
5	Average weight of strip with releasing paper only (g)	0.236 (0.220 –0.300)
6	Wound pad length (only) (mm)	24 ±1
7	Wound pad width (only) (mm)	13±1
8	Bandage Length (without releasing and packing paper)	72±1
9	Bandage Width (without releasing and packing paper)	19±1

Impregnation of wound pad

After getting solution, next step was to impregnate the wound pad with it, which involves following stages.

Impregnation machine process and behavior

Rim/ wound Pad roll: 6 non-impregnated rolls/ rims easily installed on Impregnation machine rollers and easily connected by stretching and fixing them on the other side of rollers. Switched on impregnation machine and adjusted the machine as per below specification: (Air flow control: 3 inches, squeezer gauge: 5 mm, speed: F40.45 Hz and thermostat temperature: 170 to 180°C).

Product (solution) holding pocket/ duct filled easily neemplast solution in the pocket deep up to desired level. Filled the Neemplast herbal solution in the solution duct and started the impregnation process, the wound pad rollers started moving and dipping or soaking into the solution by aid of a moving arm fix at the top of solution duct. After dipping and squeezing the wound pad reaches in to drying chamber for drying. End or receiving roller were moving well and receiving the soaked, saturated and dried impregnate wound pads.

Medicated plaster shape cutting and packing machine process and behavior

Fixed the dried impregnated rolls, release paper, printed and unprinted paper packing rolls on plaster shape cutting and packaging machine and started the process. Plaster shaping was done smoothly by cutting wound pad roll according to the pre-defined specification.

Paper packing and positioning was done accurately in connection with plaster shaping. Sealing and cutting were done nicely by push- punch type cutter. Optical test were performed on finished strips under white florescent light and found all of them centrally aligned.

Quantitative and microbiological test conducted on bulk and finished products and found satisfactory results. Finished product (bandage) specifications and flow chart of the process by using impregnation and medicated plaster shape cutting and packing machine as shown in Table 2 and Figure 1.

Evaluation of formulations

Physical evaluation (appearance)

Light yellow to colorless transparent liquid with characteristic odor were checked visually.

pH

pH of aqueous solution of the formulation was measured by calibrated Hanna pH meter (HI 110).

Weight per ml

It was determined by pycnometer at the specified temperature (24^{o}C).

Quantitative estimation of eugenol in neemplast by high performance thin layer chromatography (HPTLC)-densitometry

The quantity of Eugenol in Neemplast was observed by using HPTLC-Densitometry. In this process the equipment CAMAG Scanner III, CAMAG Linomate 5 or Equivalent and HPTLC silica gel G60F_{254} were used. It also included solvent system named n-Hexane: Ethyl acetate: formic acid (8:2:0.1) with wave length: 254 nm. For this anylsis, there were two steps involved as given below:

Standard preparation

Prepared standard solution by dissolving 10 mg Eugenol standard and 5 ml of methanol in 10 ml volumetric acid flask with continuously shaking. The volume was adjusted upto the mark with methanol.

Sample preparation

Weighed about 1.0 gm wound pad of Neemplast in 100 ml conical flask, added 10 ml of methanol quantitatively by using graduated pipette, covered it properly to avoid evaporation of solvent. Stirred it

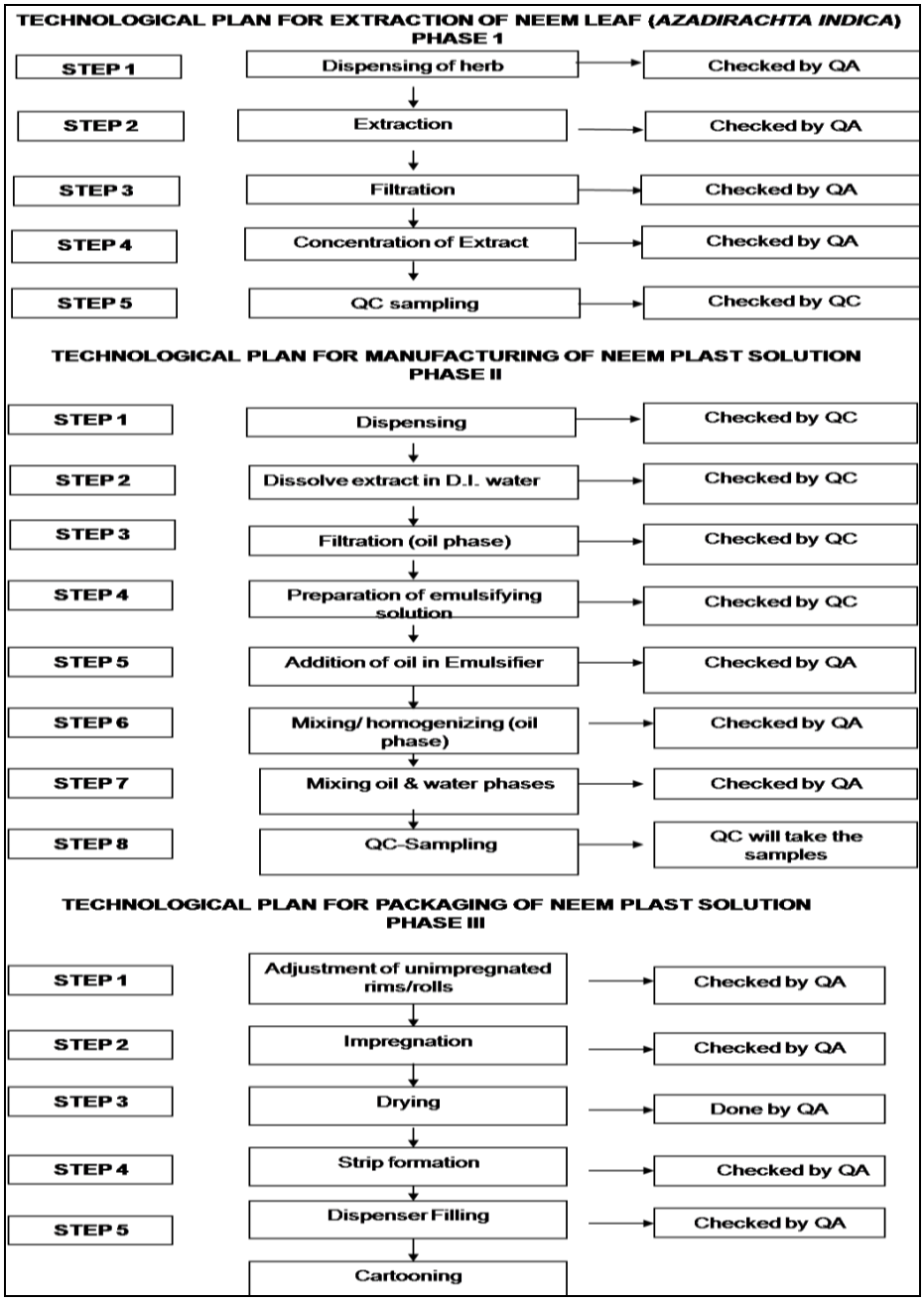

Figure 1. Flowchart of Neemplast development.

for 20 mintes, sucked the solvent through dropper or pipette and filtered the solution carefully through Whatman filter paper No. 44 and used filtrate as sample for sample application.

TLC preparation

Performed analysis on 10 × 10 cm HPTLC silica gel G60F_{254} plates with fluorescent indicator. Before started the analysis, HPTLC plate was cleaned by predevelopment with the methanol by ascending method. (Note: Immerse HPTLC Plate in a CAMAG glass chamber (20 x 10 cm), contains 30 ml methanol (HPLC grade) as solvent system cover the chamber with glass lid and wait to develop the plate to the top with methanol. After complete development, removed the plate from TLC glass chamber and dry it in an oven at 105°C for 5 min).

Application procedure

Applied three spots of 10 µl (in the form of band) of standard preparation along with three spots of 10 µl of sample preparation as the bands on the same plate by means of a CAMAG Linomat 5 (automated spray-on applicator equipped with a 100 µl syringe and operated with the settings band length 6 mm, distance between bands were 14 mm, distance from the plate side edge was 15 mm,

and distance from the bottom of the plate was 15 mm). After sample application dried the plate in hot air oven for 5 min at 105°C.

TLC development

Developed the plate by immersing sample HPTLC Plate in a CAMAG glass chamber (20 x 10 cm) contained the solvent system (n-Hexane: Ethyl acetate: Formic acid (8: 2: 0.1)), wait to develop the plate to a distance of 8 to 9 cm. After complete development, allowed the plate to dry by keeping in open air for one day.

TLC scanning

Scanned the plate next day in the densitometer by linear scanning at 254 nm by use of a TLC Scanner III CAMAG with a mercury lamp, and integrate the area of the spots corresponding to Eugenol standard as show in Figure 3. Calculated the amount of Eugenol in mg per capsule by following formula.

$$\text{Content of eugenol} = \frac{A_{SMP} \times W_{STD} \times \text{dilution of SMP} \times P \times W_{AVG}}{A_{STD} \times \text{dilution of STD} \times W_{SMP} \times 100}$$

Where, A_{SMP}, Avg. area of sample; A_{STD}, Avg. area of standard; W_{STD}, weight of standard, mg; W_{SMP}, weight of sample, g; P, percent purity of standard; W_{AVG}, average weight of neemplast wound pad.

Microbial assay

The antimicrobial activities of different formulations were determined by tryptic soy agar (TSA), tryptic soy broth (TSB), lactose and peptone water test tubes. In this method, stock American Type Culture Collection (ATCC) culture of *Bacillus subtilis* (British and Americal Pharmacopoea) was incubated in TSB for 3 -5 days. When growth occurred streak on Petri plates containing TSA media in order to get isolated colonies, incubated it at 32 $\pm$ 2.5 C for 1 day. After incubation period, took heavy loop full of cultures and inoculated it into test tube containing peptone water that was dedicated for that particular organism. Next step was to make serial dilution in peptone water test tubes from 1: 10 to $1:10^8$. Then took 1 ml from each tube and transferred it into Petri plate that was specifically mark for that particular organism. Then poured 15-20 ml TSA in each plate and incubated for 3 days. Kept all the test tubes in refrigerator. After incubation period read plates and counted number of colonies forming unit at each dilution factor. All results were recorded. For comparison of test sample (Neemplast) and control sample (Sani-plast) culture of *B. subtilis* from TSB agar poured into melted TSA agar that is, (40 - 45°C). The plates were allowed to solidify. Incubated all the plates for 24 h. The test was performed in duplicate. Next day took out all plates and placed Neemplast strip on Petri plate with the help of sterile for escape, after that incubated all these plates for 2 – 3 days. The antibacterial activities were observed by measuring the zone of inhibition (mm) of both the Neemplast and Sani-plast with the help of Vernier Caliper. Recorded the results and compared zone of inhibition of sample with standard (Sani-plast-Acrinol) as shown in Figure 2. On the basis of the observations we can conclude that the result was satisfactory. The prepared dosage form of Neemplast was evaluated for various evaluation parameters such as general appearance, and weight variation, antimicrobial activity. The final formulation found to have light yellow to colorless transparent liquid with characteristic odor and had pH 4.5-6.5 with average weight of 0.5226 g ± 5% (0.494 to 0.546 g).

RESULTS AND DISCUSSION

Neemplast was evaluated in terms of appearance, and found light yellow to colorless transparent liquid with characteristic odor were checked visually. pH of aqueous solution of the formulation was also measured and weight per ml was determined by pycnometer and with the help of HPTLC-Densitometry the active (Eugenol) of Neemplast was determined. By analyzing antimicrobial activity it was found that newly developed herbal antiseptic wound pad Neemplast had an inhibitory effect on the *B. subtillus*. It also showed satisfactory zone of inhibition compared with control sample. Neemplast is based on natural source and showed zone of inhibition very close to Acrinol based Sani-plast, so we can conclude that Neemplast has similar efficacy and safe to use as compared to synthetic (Acrinol based) Sani-plast. In this way Neemplast solution and finished formulation showed comparatively satisfactory antimicrobial activity than control sample.

The present study shows that newly developed polyherbal anti septic wound pad was successfully designed, developed and assessed its activity by antimicrobial activity against control acrinol based Sani-plast. In addition, the quantity of active ingredients eugenol in Neemplast was determined by using HPTLC-Densitometry. Hence herbal wound pad could be used as better and safe substitution of synthetic wound pad Sani-plast. In previous studies it is described that when leaf of Neem are boiled Neem leaf in water, it serves as an excellent antiseptic to clean the wounds, soothes the skin, swellings and also eases skin problems (Shahidi et al., 2004). When applied against Gram-negative and Gram-positive microorganisms, the Neem oil from the leaves, seeds and bark have shown a wide spectrum of antibacterial activity (Chopra et al., 1956). It is reported that Neem oil has the antimicrobial activity against a variety of pathogens like *Vibrio cholerae, Klebsiella pneumoniae, M. tuberculosis* and *M. pyrogenes* in vitro study (Satyavati et al., 1976). The extract of Neem has shown antibacterial activity against *Escherichia coli, Klebsiella pneumoniae* and *B. subtilis* (Jagannadh and Radhika, 2006; Shravan et al., 2011). The leaves of Neem are locally applied on skin as poultice for curing boils, and also used in as antiseptic for healing and cure of wounds, ulcers and eczema. Traditionally it is said that Neem leaves are used to treat itching and other skin diseases, and even bathing with Neem leaves has shown beneficial effects to cure skin ailments (Ghani and Khazainul, 2004; Chatterjee and Pakrashi, 2011). Against *B. subtilis* methanol extract of *Azadirachta indica* showed marked activity (28 mm) (Shravan et al., 2011). Active ingredients of Neem works in wound healing process and also help the skin to retain its agility as the wound heals (Pandey et al., 2011). It contains terpenoids that helps wound healing (Hawkins and Ehrlich, 2006). Eucalyptis oil has wound healing properties mainly due to monoterpenes (Sarkar, 1994). Oil of eucalyptus has been

a

ANTIMICROBIAL ACTIVITY OF NEEMPLAST

(1) Sample Neemplast

(2) Control Sample Sani-Plast (As Standard Use)
(Active-Acrinol)

Germicidal Activity:

The Following Pathogenic Organisms *Bacillus Subtilis* are used against the Product (Neemplast).

Organisms:

BACILLUS SUBTILIS = 4000 Organisms

Sample Neemplast (As Test Sample)	Control Sample Sani-Plast (As Standard Use)
No-Growth	No-Growth
No-Growth	No-Growth
No-Growth	No-Growth
No-Growth	No-Growth

b

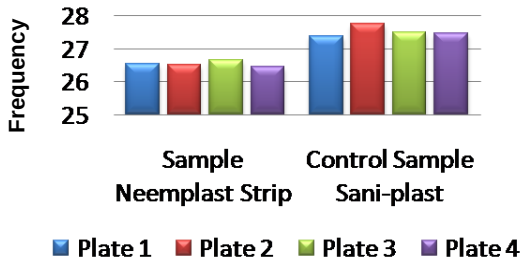

Figure 2. (a) Microbiology report No growth was observed for neemplast; (b) graphical representation of antimicrobial activity of test (Neemplast) and control (Sani-plast) sample.

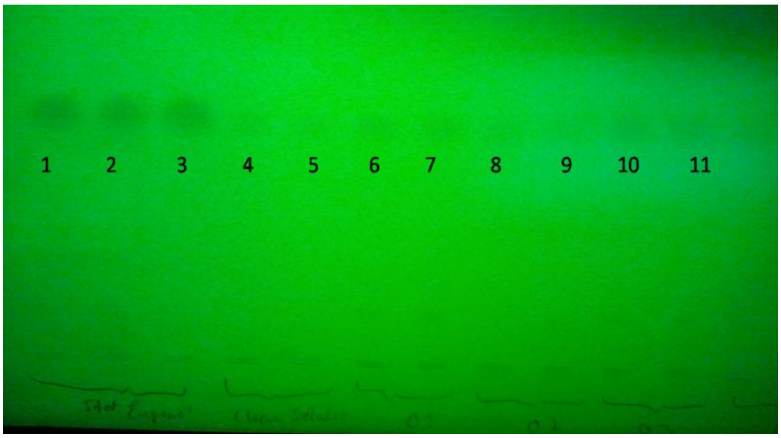

Figure 3. Quantitative estimation of eugenol in neemplast by HPTLC-densitometry**.** 1, 2 and 3, eugenol reference standard; 4 and 5, neem plast bulk solution; 6 and 7, wound pad 0; 8 and 9, wound pad 02; 10 and 11, wound pad 03.

traditionally used in Ayurveda as an antiseptic and also it has reported that this oil has antibacterial action (Nadkarni, 1979). Topical application is effective against different microorganisms and also reported antibacterial action (Sherry et al., 2001; Kumar, 1988; Ahmad and Beg, 2001). The prepared dosage form of Neemplast was evaluated for various evaluation parameters such as general appearance and weight variation, antimicrobial activity and quantitative analysis. The prepared bandage consist of sterilized non-woven (70% viscose, 30% polyester) antiseptic pad impregnated with Neem and Black pepper (Patani, 2002) distillate along with Clove and Eucalyptus oil (Usmanghani et al., 2009) which is covered by releasing and printed paper. The final formulation found to have light yellow to colorless transparent liquid with characteristic odor and had pH 4.5

to 6.5 with average weight of 0.5226 g ± 5% (0.494 to 0.546 g).

Conclusion

Herbal dosage forms of *Neemplast* showed good elegance and appearance. It is an excellent effort to design and develop the herbal antiseptic wound pad having satisfactory zone of inhibition and antimicrobial activity comparable with control sample Sani-plast. By using HPTLC-Densitometry Eugenol (Neemplast) was detected in comparison with Acrinol (Sani-plast). This study revealed that the developed herbal wound pad was suitable dosage form for antiseptic bandages.

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