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Herbal Coated Natural Nonwovens for the Development of Foot Insoles With Reference To Diabetic Foot Ulcer

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Abstract: ***Diabetic foot problems, such as ulcerations, infections, and gangrene, are common among diabetic patients. Diabetic patients with foot ulcers are advised to use a rigid insole. In the present study, plant extracts were studied for their antibacterial activity. The extract derived from M. pubescens exhibited potent antibacterial activity against the test pathogens. Natural nonwovens coated namely cotton and Bamboo was coated with the methanol extract of Morinda pubescens and the antibacterial activity of the coated nonwovens were determined by ENISO method and it was found to possess potent antibacterial activity against the test pathogens. The GC-MS analysis of the extract indicated the presence of various photochemical which are reported to have wound healing property, odour control etc. The wound healing property of the methanolic extract determined by wound scratch assay using L 929 fibroblast cell lines. Rapid initiation of wound healing process in 18 hrs was observed.***
Keywords: Morinda pubescent, methanol extract, natural nonwoven, antibacterial activity, wound healing assay.

I. INTRODUCTION

Foot wound is a major problem in diabetic patients. They have about 25% chance of developing a foot ulcer in their lifetime and about half of which are clinically infected [1]. The pathophysiology of foot infections in persons with diabetes is complex due to neuropathy, arteriopathy and other factors like microbial virulence, antibiotic-resistance and microbial load of secondary infection causing pathogens of foot ulcers [2]. Patients having foot ulcer with a transcutaneous oxygen pressure value < 30 mmHg use an appropriate shoe with a rigid insole for pressure relief [3]. The currently available insole in market reduces the stress in knee joints and reduces foot problems [4]. But these insoles are expensive and do not possess wound healing property. The synthetic chemicals or drugs used to treat diabetic foot ulcer may raise the cross reactivity inside the body and widely inhibit bio-membrane functions. In addition to the above facts, treatment of foot wound in diabetic patients has become a challenge due to the prevalence of antibiotic resistant pathogens like Methicillin - Resistant *S.* aurous (MRSA) [5]. India being a tropical country with rich flora, exploring plants for the development of novel wound healing agents will be an effective alternative strategy to combat the problems with the existing drugs. Traditional medicine is also cost effective when compared to modern medicine. Active principles from plants have been reported to possess microbicidal, pesticide and fungicidal property and are incorporated in the pharmaceutical drug formulations [6].

Application of natural products from plants for wound healing has a long history and there is growing interest in the use of natural products for the treatment of ailments. The objective of the current study is the development of herbal wound healing material to be used as an insole and wound dressing material. As natural nonwovens are most suitable for medical applications, natural nonwovens namely cotton nd bamboo is taken for the study. Three plants namely Morinda pubescens, Cleome viscoses and Eupatorium odoratum have been screened for antibacterial activity against Methicillin resistant Staphylococcus aureus (MRSA), Enterobacter cloacae, Klebsiella pneumonia, Proteus mirabilis and Escherichia coli. The wound healing property of the extract has been studied and the active principles present in the extract were identified by GCMS. The selected herbal extracts were coated on to the nonwovens and the antibacterial activity was assessed by ENISO method. The finished cotton and bamboo non woven were evaluated by FTIR analysis.

II. METHODOLOGY

A. Procurement of Materials

Natural nonwovens were selected for the current research. Cotton and bamboo non woven are purchased from Chennai, Tamil Nadu, India. The herbs selected for the study namely *M. pubescens, C.* viscose and E. odoratum were collected from herbal garden, RND herbals, Coimbatore. Five pathogens known to be responsible for diabetic wound infection (MRSA, Cloacae, K. pneumonia,

P.mirabilis and E. coli) were purchased from MTCC IMTECH, Chandigarh, India. The solvent Methanol employed for extraction was AR Grade and was purchased from Hi Media, Mumbai.

B. Extraction of herbs

The leaves of all the three herbs were shadow-dried for two weeks at room temperature and powdered in a blending machine. 8 g of the plant material is placed inside a thimble made from thick filter paper, which is loaded into the main chamber of the Sechelt extractor. This extractor is placed onto a distillation flask containing the solvent. The soxhlet is equipped with a condenser, and 100ml of the solvent is heated to reflux. The warm solvent vapour travels up a distillation arm and floods into the chamber housing the thimble. When the chamber is almost full, it gets automatically emptied by a siphon side arm back down to the distillation flask. This cycle was allowed to repeat many times so that the desired compound gets concentrated in the distillation flask. The crude methanol extract thus obtained was stored at 4 ^{o}C for further studies [7].

C. Screening of herbs by well diffusion

The antibacterial activity of the different plant extracts was evaluated by Agar well diffusion method. Sterile Muller Hinton Agar plates were prepared. The plates were allowed to solidify for 5 minutes and wells of 6 mm were punctured using a well borer. Log phase overnight cultures (0.1ml) of the bacterial suspension namely S. aurous and *E.* cloacae were swabbed uniformly over the surface of the agar. 10,20,30,40 and µl of each herbal extract was loaded separately in different wells and the plates were kept for incubation at 37ºC for 24 hours. The antibacterial activity was evaluated in terms of Diameter of zone of inhibition, measured and recorded in millimetres [8].

D. Finishing to fabrics

Finishing was done by dip and dry method. The selected plant extract was taken in a tray and the selected cotton and bamboo non-woven fabric was dipped in the extract for two hours. The fabric is shade dried and then tested for antibacterial activity against diabetic wound pathogen [9].

E. Antibacterial assessment of the finished fabric (EN ISO 20645 Test method)

The antibacterial activity of the finished fabrics was tested according to EN ISO 20645 against MRSA, E. cloacae, K. pneumonia, P. mirabilis and E. coli. Muller Hinton Agar plates were prepared by pouring 15 ml of media into sterile Petri dishes. The plates were allowed to solidify for 5 minutes and 0.1% inoculum was swabbed uniformly and allowed to dry for 5 minutes. The finished fabric with the diameter of 2.0 ± 0.1 cm was placed on the surface of medium and the plates were kept for incubation at 37 ºC for 24 hours. At the end of incubation, the Diameter of zone of inhibition formed around the fabric was measured in millimetres and recorded [10].

F. In vitro Wound Scratch Assay of plant extract

L929 mouse fibroblast cells were grown in 24 well plates at a density of 1 x 10^5 cells/ml and cultured until ~ 80 % confluence. A small linear scratch was created in the confluent monolayer by gently scraping the layer with sterile cell scrapper [11]. Cells were thoroughly rinsed with 1 X PBS to remove cellular debris and treated with 25 µl of methanolic extract of the selected plant. Cell proliferation was monitored at different time intervals: 0, 24, and 48 hrs and images of the migrated cells were taken at all different time points using digital camera (Nikon, Tokyo, Japan) connected to the inverted phase contrast microscope (Radical instruments, India). Extent of wound healing was determined by the distance traversed by cells migrating into the denuded area.

G. Analysis by Gas Chromatography/Mass Spectrometry (GC/MS)

Gas chromatography-mass spectrometry (GC-MS) analysis of the methanolic leaf extract was performed on a GC-MS equipment (Thermo Scientific Co.) Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II. The Gas Chromatography/Mass Spectrometry (GC/MS) instrument separates chemical mixtures (the GC component) and identifies the components at molecular level (the MS component). The GC works on the principle that a mixture will separate into individual substances when heated. The compounds present in the methanolic extract of *M.* pubescens were separated by GC/MS and identified by comparing the spectrum of the separated compounds with the known mass spectra of several thousand known compounds stored in the database.

H. Characterization of Herbal extract loaded cotton and Bamboo non-woven Fabrics

Different functional compounds absorb infrared radiation at frequencies corresponding to their own molecular vibration frequencies [12, 13]. The radiation emitted is in the vibrational IR region and also referred in terms of wave numbers (ν), which is the number of waves per centimetre. IR radiation is electromagnetic radiation with frequencies 400 – 4000 cm^{-1}. Fourier Transfer Infra-Red (FTIR) spectrophotometer (Shimadzu, Japan) was performed to analyse the functional groups of the compounds loaded in the fabrics employed for the investigation.

III. RESULTS

Various plant products have been used in the treatment of wounds over the years as wound healing is a complex and protracted process of tissue repair. Herbal extract used in this study promotes healing of wounds and fight against infection. In the present research, the crude extracts were derived from M. pubescens, C. viscose and E.odoratum using methanol as a solvent (Fig. 1 and 2).

Eupatorium odoratum Morundah pubescent Cleome viscose

Fig.1.Selection of herbs

Fig.2. Solvent extraction by soxhlet apparatus

The results (zone of inhibition) of antibacterial activity by well diffusion method are tabulated in Table 1. The results revealed that the extract of M. pubescens was found to exhibit potent antibacterial activity against the microorganisms studied.

Table 1: Antibacterial activity by well diffusion method

S.no	Herbal extract	Organisms	Zone of inhibition (mm)				
			Concentration (µl)				
			10	20	30	40	50
1.	C. viscose	S. aureus	0	8	11	12	13
		E. cloacae	0	0	0	0	0
2.	M. pubescens	S. aureus	0	0	0	11	12
		E. cloacae	0	16	14	17	17
3.	E. odoratum	S. aureus	0	0	0	10	11
		E. cloacae	0	0	0	0	0

The diameter of zone of inhibition was measured as 12 ± 0.22mm and 17 ± 0.32mm against *S. aureus* and *E. cloacae* respectively Fig. 3 A and B.

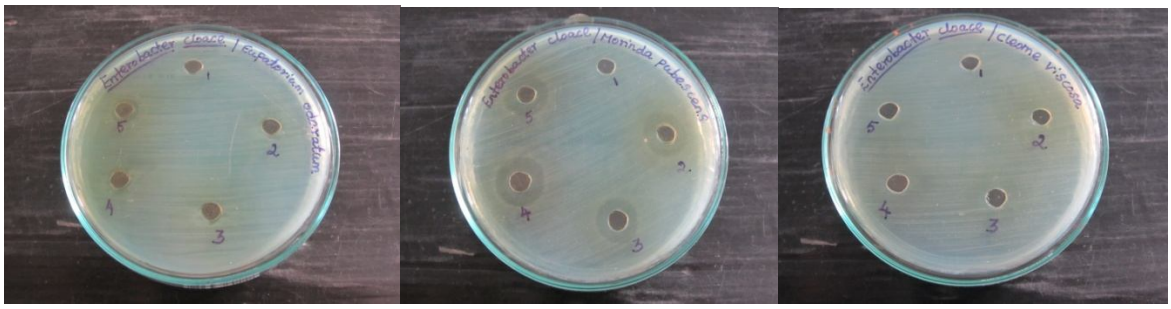
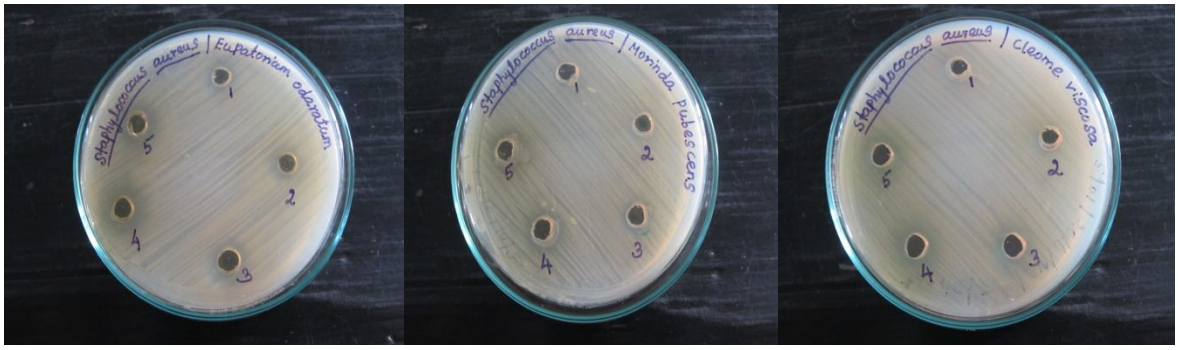


Fig. 3. Antibacterial activity by well diffusion. (A) Activity against *E. cloacae*



(B) Activity against S. aurous

The zone of inhibition exhibited by the selected M. pubescens extract coated fabrics determined by ENISO 20645 is tabulated in Table 2 and Fig. 4.

Table 2: Antibacterial activity by EN ISO 20645 method

S. no.	Name of the sample	Name of the fabric	Methicillin resistant S.aureus	E. cloacae	K. pneumoniae	P. mirabilis	E. coli
1	Methanolic extract of M. pubescens	Bamboo	33	30	31	33	42
		Cotton	32	35	32	39	40

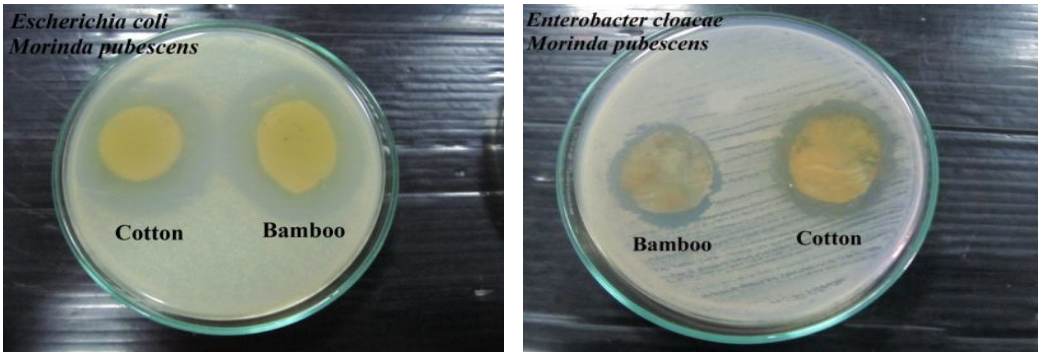


Fig.4.Antibacterial activity by EN ISO 20645

Metabolic extract (50µg/mL) of M. pubescens initiated the wound healing in L 929 fibroblast cell lines after 18hrs. It was observed that gradual increase in wound healing was noticed in 24 hrs with 100 µg/mL of the extract. The wound healing was almost 2 times higher in cultures treated with 100 µg/mL of herbal extract, when compared to the values obtained in the control group. Methanolic extract of M. pubescens showed 95% of wound healing activity with 50 µl & 90% of healing activity with 75 µl & 100 µl of extract at 48 hrs as compared to the control (Fig. 5).

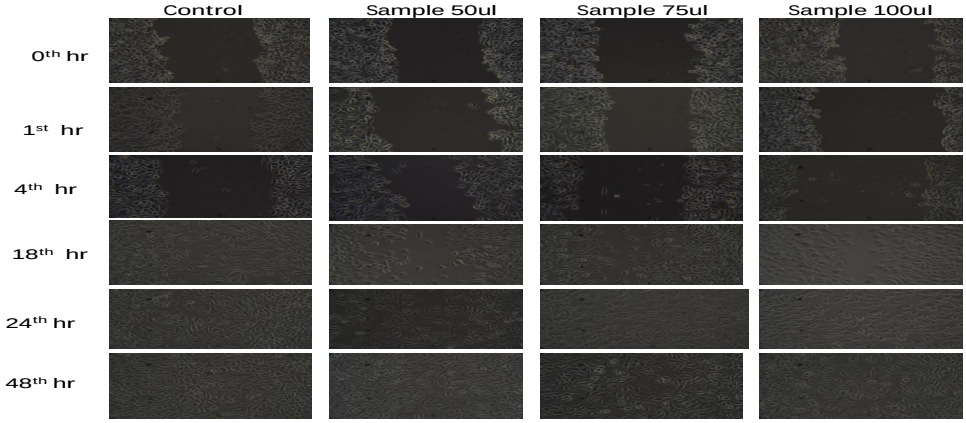


Fig. 5 Wound scratch assay of *M. pubescens*

A. Analysis by GCMS

The mass spectrometer analyses the compounds eluted at different times to identify the nature and structure of the compounds. The GCMS chromatogram is shown in fig. 6.

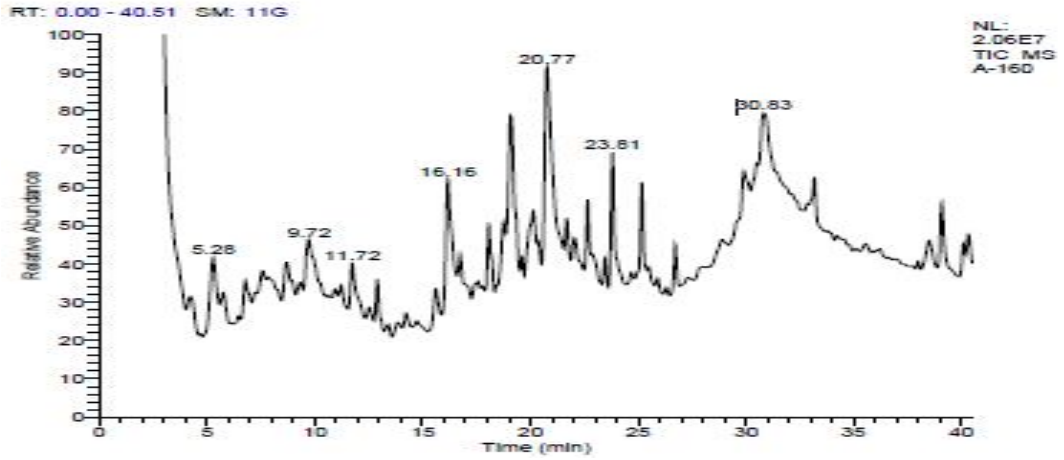


Fig 6. GCMS Analysis of M. pubescens

The mass spectra of the compounds were compared with the data library and the identified compounds are listed in table 3. GC-MS analysis of the methanolic extract of M. pubescens showed the presence of major compound namely isochromene, a derivative of benzopyran used to treat diabetes and also used for smooth muscle relaxation. The compound isoquinoline separated and identified has been reported to be used as topical antiseptic, antifungal agents and also can be used as anaesthetics [14]. The other compounds identified namely isopropylidene can be used in the treatment of vascular endothelial cell injury [15]. Sarcocapnine, an isocularine alkaloids having diverse and important physiological effects on humans [16]. Thus, all the constituents present in the extract have been identified to play a major role in various aspects of aspects of wound healing. The library search results that received from the GCMS analysis of M. pubescens extract is tabulated in Table 3.

Table 3: Library search results of GCMS analysis of *M. Pubescens*

SI	RSI	Compound name	Probability	Molecular formula	Molecular weight	Area%
459	758	1-Methyl-3-(3,4-dimethoxyphenyl)-6,7-dimethoxyisochromene	26.17	C20H22O5	342	1.42
456	691	4,5,6,8-PTetramethoxy-2,3-dihydroindeno[1,2,3-ij]isoquinolin-9-ol	23.12	C19H19NO5	341	1.42
438	660	9H-pyrrolo[3',4':3,4]pyrrolo[2,1-a]phthalazine-9,11(10H)-dione,10-ethyl-8-phenyl	11.92	C21H15N3O2	341	1.42
442	662	4,5,6,8-Tetramethoxy-2,3-dihydroindeno[1,2,3-ij]isoquinolin-7-ol	6.86	C19H19NO5	341	1.42
419	613	2,3,5,6-Tetramethoxydibenzo[f,hi]indolizine-11-one	6.06	C19H19NO5	341	1.42
415	605	3-(4'-Methoxyphenyl)-1-acetyl-2-phenylindolizine	5.12	C23H19NO2	341	1.42
400	635	3-Formyl-N-methyl-9-[phenylethynyl]dibenzo[2,3-a : 5,6-a'] (1,4)-thiazine	3.10	C22H15NOS	341	1.42
397	587	3-à-Pyrrolidino-17a-aza-D-homoandrosta-5-ene	2.74	C23H38N2	342	1.42
388	583	SARCOCAPNINE	1.99	C20H23NO4	341	1.42
383	532	Ethyl(E)-N-(tert-butoxycarbonyl)-2,3,4,7-tetradeoxy-4,7-imino-5,6-isopropylidene-L-ribo-hept-2-enonate	1.60	C17H27NO6	341	1.42

B. Analysis by FTIR

The FTIR analysis confirms the presence of alkaloids, phenols and glycosides present in the methanolic extract of M. pubescens finished fabric. FTIR was adopted to compare and characterize the potential interactions of photo-constituents present in the extract with that of cotton and bamboo non oven. FTIR spectra of the M. pubescens leaf extract finished bamboo and cotton fabric is shown in fig. 7 and 8.

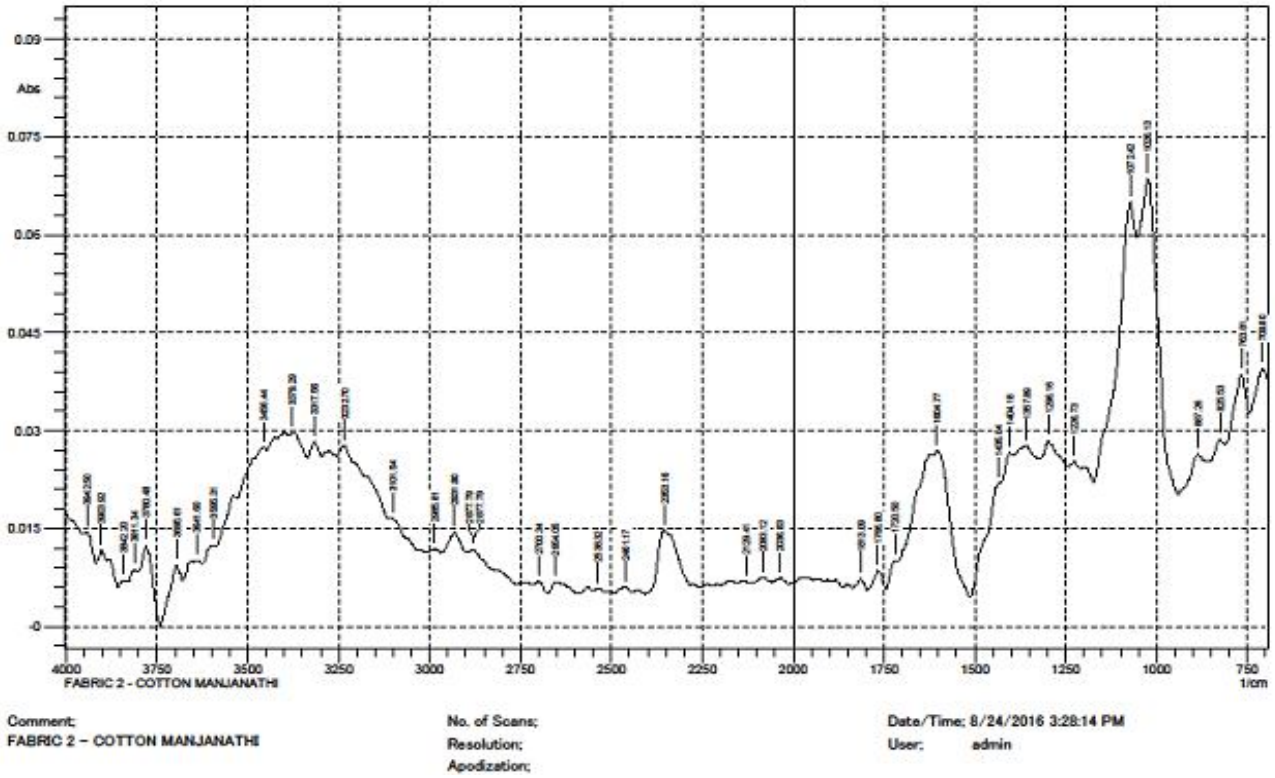


Fig.7. FTIR analysis of Finished Cotton Fabric

The spectra of M. pubescens leaf extract finished bamboo fabric show identical peaks and bands which is an evidence of the herbal finish on the bamboo fabric. The details of the peaks and bands are shown in (Table 4 and 5).

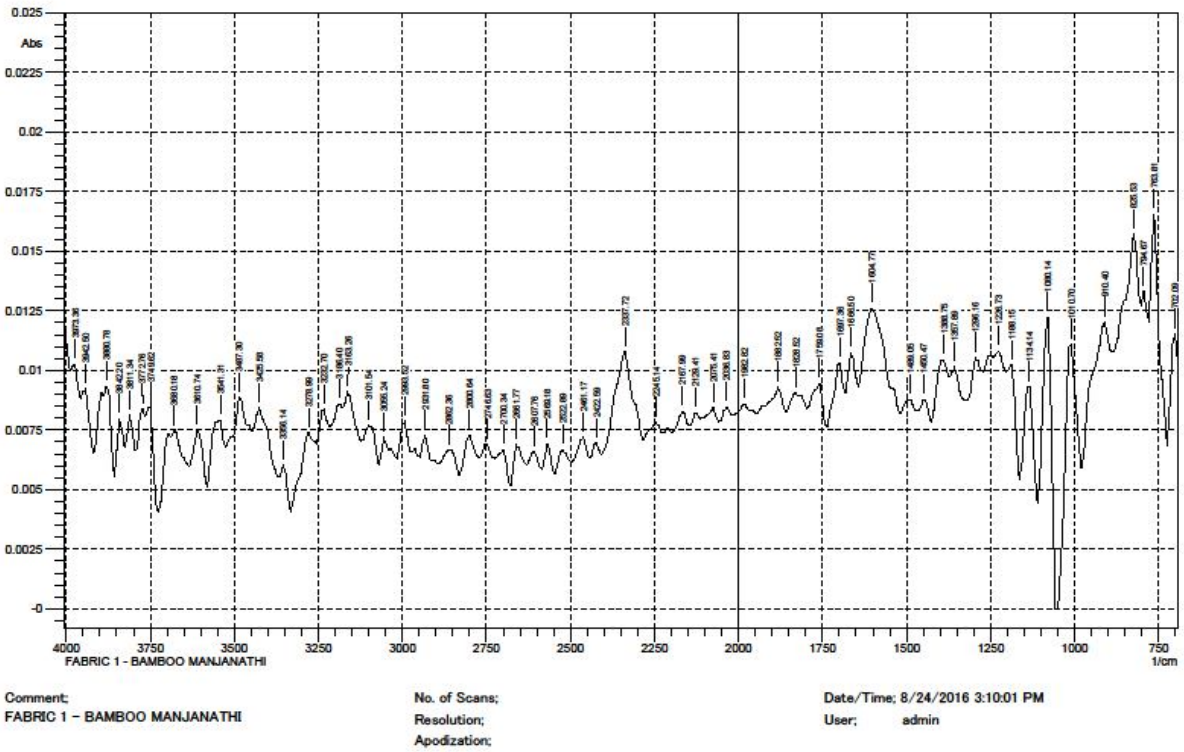


Fig.8. FTIR analysis of Finished Bamboo Fabric

Table 4: M. pubescens leaf extract finished bamboo fabric

S.no	Wave number (cm^{-1})	FTIR spectral distribution	Intensity
1	3300	N-H primary and secondary amines and amides	M [a]
2	1666.50	C==O in tertiary amines	V [b] S [c]
3	1080.14	C-O stretching vibration	S [c]
4	2800 & 2746	CH stretching modes – Ch_3 attached to O or N	M [a]
5	1759.08	C==O stretch vibration of aldehyde	S [c]
6	1300.75	C-N stretching in amine	M [a]

[a] Medium
[b] Very
[c] Strong

Table 5: M. pubescens leaf extract finished Cotton fabric

S. No	Wave number (cm^{-1})	FTIR spectral distribution	Intensity
1	1150	S=O symmetric stretching	M [a]
2	1350	S=O symmetric stretching	V [b] S [c]
3	1550	N-H bending	S [c]
4	1750	C=O stretching	M [a]
5	750	S-O stretching	M [a]

[a] Medium
[b] Very
[c] Strong

IV. DISCUSSION

In current scenario, medical textiles play a vital role in hospital applications. Wound dressing materials are found to be intimate with the patients and so it is important to provide the dressing material as barrier against microorganisms and as a suppressor of blood odour. In the present investigation, the functional finishes imparted show antimicrobial activity. Wide range of natural and synthetic manmade fibres with better comfort properties and anti-microbial properties have been introduced commercially [17].

Wound healing is the general repair response of the body directly after the disruption of skin integrity. This repair mechanism mainly composed of five interconnected and overlapping phases; haemostasis and inflammation, neovascularization, granulation, reepithelization and remodeling [18]. People's consciousness towards traditional medication has also changed and is very encouraging. The concept of moist wound healing has been well accepted and traditional medication has also integrated this method to fasten the healing process. Several studies using herbal and traditional medicine from different continents have been predicted in wound care management [19].

The antibacterial activity of E. odoratum shows good result against some of the pathogens but it is relatively small when compared with that of M. pubescens. Also E. odoratum possess high analgesic property than antibacterial activity. The same has been reported by Venkataraman et al. [20]. Hence, E. odoratum can be used in preparation of polymer with several mixtures of folklore medicine as a cost effective and safe medication. The antibacterial activity of T. procumbens was found to be comparatively lower than the other extracts employed in the study. In 2011, Pai et al. reported the use of T. procumbent as a component in the formulations for the treatment of nosocomial infections caused by P. aeruginosa [21]. The hepatoprotective properties of M. pubescens fruit extract against D-galactosamine (D-GalN)-induced liver injury in rats has been evaluated and reported by Surrendering and Mathivanan [22].

The preliminary phytochemical analysis of fruit extract indicated the presence of phenols and alkaloids. In this study the methanolic leaf extract of M. pubescens shows greater activity towards wound pathogens. Earlier studies on M. pubescens reported the high antioxidant potential for the leaf extract and it has been identified that the activity is due to the combined effect of

the individual phytochemicals present in the extract. The antioxidant activities of the individual compounds are reported as : Hyoscyamine > campesta-5-22- trien-3-ol > β-sistosterol > stigma steroid > ergosteroid > stigmasta-4-en-3-one > stigmasta-4-22-dien-3-one > E-phytol [23]. It has been identified that the plant increases not only lysyl oxidase but also, protein and nucleic acid content in the granulation tissue, probably due to its high glycosaminoglycan content [24].

The two important events necessary for wound healing is cell proliferation and cell migration [11]. We have chosen L-929 cell line which was an established and well-characterized mammalian cell line that has demonstrated reproducible results. These fibroblast cell lines were selected for the study as fibroblasts play a major role in wound healing process, besides keratinocytes. Test formulation was prepared with the methanolic leaf extract of M. pubescens which is known to have several medicinal properties including potent antioxidant and cytotoxic activity [23]. Fibroblast is the main cell architect participating in this process of healing by contraction, which is also involved in the synthesis and deposition of the extracellular matrix. Hence, the fibroblast *in vitro* model is integral to correlating the contractile events of wound healing [25].

The characterization of methanolic leaf extract of M. pubescens was performed by GC-MS. This type of GC-MS analysis is step towards understanding the nature of active principles in this medicinal plant, and this type of study will be useful for further detailed study. Isochromene, a derivative of benzopyran was used in the treatment of various diseases including hypertension, cardiac ischemia, arrhythmia, smooth muscle relaxation, diabetes, cardio-protective and anti-angiogenic activities [26]. Dihydroindenoisoquinolines have been examined for their cytotoxicity in cancer cell cultures [27]. Isopropylidine are used in the treatment of vascular endothelial cell injury [15]. Sarcocapnine which is present as a minor compound also has been reported to have positive effect on physiological effects of humans. Its chemotaxonomic studies were also reported by Suau et al. [16]. During 21st century, bamboo pulp fiber is widely employed as a natural, green and eco friendly textile material. Bamboo fiber is characterized by its hygroscopicity, air permeability, softness in feel, easy to straighten and dye ability with splendid colour effect of pigmentation. It is also a new environment-friendly fiber which is both anti-bacterial and deodorizing in nature. Bamboo fabric has good breathability due to high air permiability, and keeping cool and comfortable. This is because the cross-section of the bamboo fiber is filled with various microgaps and micro-holes; it has much better moisture absorption and ventilation [28]. Bamboo owns a unique antibacterial and bacteriostatic bio-agent named "bamboo kun" which gives the inherent antimicrobial property to the bamboo fiber. Bamboo fiber has excellent moisture regain and elongation but the wet and dry tensile strength are comparatively less than cotton fiber [29].

V. CONCLUSION

The leaf extract of M. pubescens exhibited significant wound healing activity and the Ethylhexanol present in the extract is known to remove the bad odour that comes from the wound. The results of the present investigation support the fact that cotton non-woven and bamboo non-woven can be used as effective wound healing material. Cotton fabric treated with the extract of M. pubescens showed good results when compared to bamboo nonwoven and can be employed for wound healing and also to avoid infections from microbes. Further work is in progress to design, standardize and develop insoles made of cotton non-woven coated with the extract of M. pubescens to facilitate rapid wound healing and prevention of infection in diabetic patients with foot ulcers.

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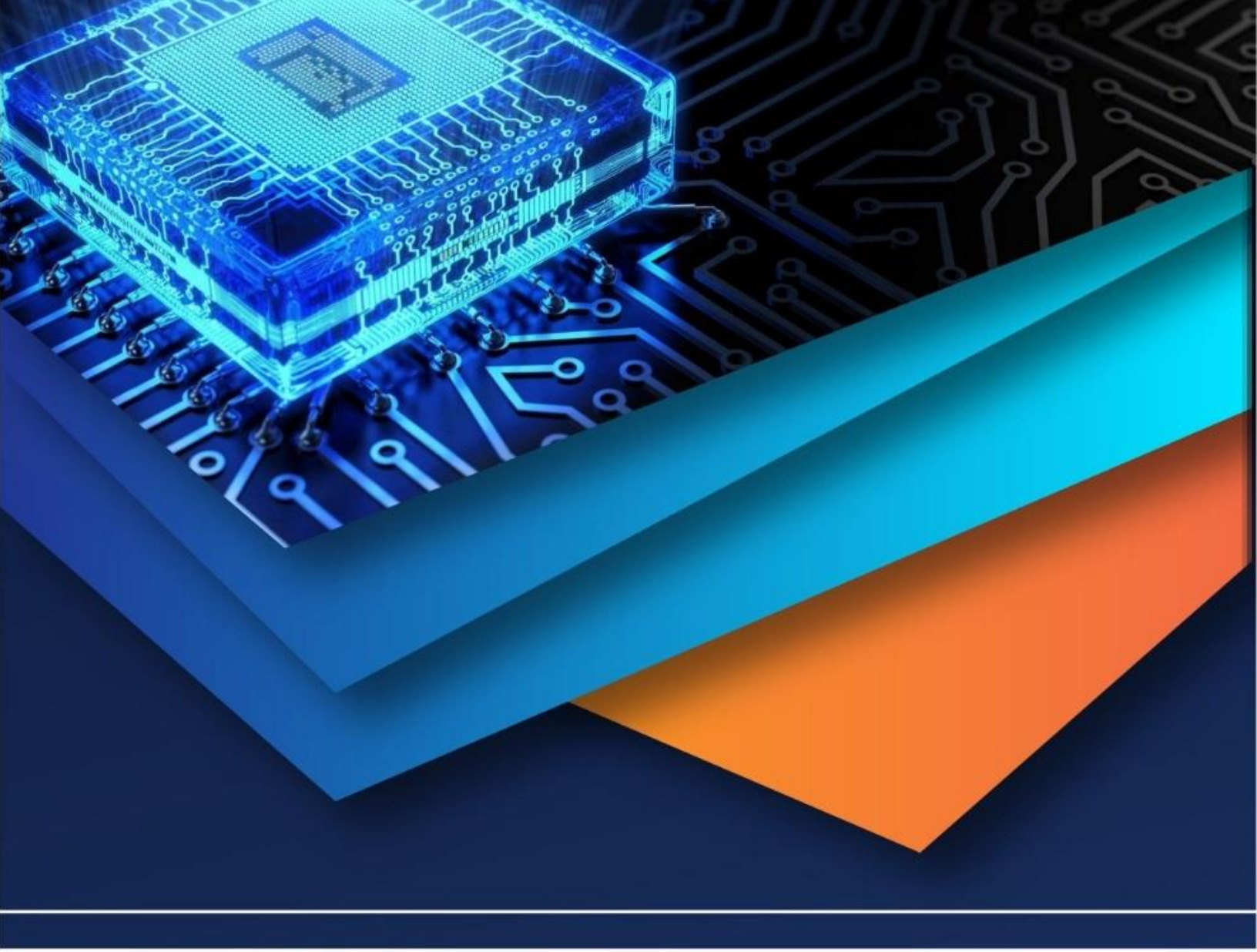

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