IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 5 Ver. I (Sep -Oct. 2014), PP 38-49
www.iosrjournals.org

Studies on Healing Activity *Vis-A-Vis* Microflora of Acute Induced Wounds against Solvent Extracts of Rhizome of *Drynaria quercifolia* Linn.

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Abstract:
Background: *Drynaria quercifolia Linn. (Polypodiaceae) is an important herbal in traditional folklore use bearing wound healing potential in Ganjam- Gajapati districts of Odisha (India). The aim of the study was to scientifically validate the folklore therapeutic claim through evaluating the healing potential of the plant rhizome extracts (methanolic and Chloroform) in normal and diabetic induced rats versus the microbial studies of isolation, characterization of different micro flora of the induced wounds and their impact in the healing process.*
Methods: *Excision and incision wounds were induced in normal and diabetic rats. 5% and 10% extracts dosage form in ointment base using standard pharmaceutical treatise, were applied and examined for the efficacy. Control groups were dressed with simple ointment base and sterile distilled water while standard groups were applied with Neosporin in normal and Mupirocin / Supirocin ointment in diabetes induced rats. Healing versus microbial infection was studied/ assessed with measurement of wound size contraction, epithelization time, leukocyte counting, isolation and identification of the micro flora in the wounds etc. Statistical analysis was performed using one way ANOVA followed by students' t - test.*
Results: *Both extracts (10%) were found to have significant healing potential evident from reduction in wound size, epithelization time and the reason were also supported by phytochemical and microbial studies. Delay in healing might be due to infection of Propionibacterium sp., Clostridium sp. and Pseudomonas aeruginosa sp. which were isolated as predominant bacterial flora in diabetic wounds contrary to Pseudomonas aeruginosa and Staphylococcus aureus that abode in the normal/induced wounds.*
Conclusion: *The folklore claim of the plant as a healing agent was scientifically validated. The tested extracts with their various phyto constituents render healing and antimicrobial property that combat and control the microbial infection in the induced wound sites at a quicker rate, was established.*

I. Introduction

A wound is a bodily injury caused by a cut, blow or other impact where the exposed dermal tissue is normally distorted or disrupted due to loss of skin integrity. It stands as a big problem in clinical practice and hence scientists and clinicians search and investigate the safety approaches to promote the healing of wounds of varied topography. Research in this direction is being undertaken across the globe to develop better healing agents providing solution to this uphill task. Wounds are of either acute or chronic type based on its etiology. External damage to intact skin results creation of an acute wound; whereas, endogenous mechanisms i.e. patho-physiological abnormalities that may lead to formation of chronic wounds. Acute wounds include minor cuts, surgical wounds, burns and abrasions etc; whereas, chronic type includes leg ulcers, foot ulcers and pressure sores etc [1]. The momentum of wound healing depends to a considerable extent on the contraction that begins within a few days after injury and prolongs for several weeks [2].

A greater look concentrated as on today is to evaluate drugs of plant origin due to their specific healing properties and action coupled with non toxic effects. Drynaria quercifolia Linn. locally (Odia) called 'Goruda' belongs to a Cryptogrammic plant group 'Pteridophyte'. It is inhabited infrequently in hill tops of Kerandimal Mountains, especially in the Taptapani-Chandragiri areas of undivided Ganjam district. There are folklore claims of its use in the treatment of wounds and cuts, where the macerated pastes of the rhizome are applied externally. Study pertains to biological activities and chemistry of the rhizome extract was reported earlier as: anti microbial[3- 6]; acute toxicity study[7]; anti-inflammatory[8]; neuropharmacological[9]; anti ulcer[10] and profiles of the isolated compounds[11]. Since, a survey of literature revealed that no detailed scientific approach has been made to study the wound healing activity of the herbal Drynaria quercifolia (DQ), the present study was undertaken to find out the efficacy of the two ointment based DQ extracts applied on induced wound models of both diabetic and normal albino rats with special emphasis on the wound micro flora. Diabetic patients are prone to infection and are at increased risk. Thus diabetic rats have disorder of decreased vascular

circulation that causes hypoxia, which induces impaired wound healing. Hypoxia intensifies inflammatory reactions and liberating increased oxidant free radicals that delay the process of wound healing[12-14]. Improper blood sugar and hypoxia from poor circulation may impair the ability of WBC to destroy pathogenic microbes (bacteria and fungi); thereby, increases infection risk[15]. Increase in blood sugar level also increases oxidant free radical and promote production of TNF α (a tumor necrosis factor) that participates in inhibition of hem-angiogenesis[16]. It is also known to be associated with variety of connective tissue abnormalities for example it causes reduced biosynthesis or accelerated degradation of newly synthesized collagen[17]. These qualitative[18] and quantitative[19] abnormalities posed to cause impaired wound healing found in diabetic cases. As local infection causes delayed or impaired wound healing, it increases susceptibility to infection by varied micro flora. Hence, an attempt to isolate the associated micro flora was under taken from the induced wounds of the normal and diabetic rats and the application of the plant extracts were tested for its efficacy and potency in controlling delayed vascularization, reduction in blood flow, decline in innate immunity and decrease in growth factor production.

II. Materials and Methods

1. Collection of the herbal and extraction of the phytochemicals: The herbal, available as an epiphyte inhabited on arboreal phorophytes or growing on rocks, was collected from the hill tops of Mahendragiri hills spread through Kerandimals of undivided Ganjam district at Taptapani - Chandragiri areas of odisha. The collected rhizomes were shade dried for three weeks and powdered mechanically for size reduction and then was subjected to successive extraction with solvents like n-Hexane, petroleum ether, chloroform and methanol, and finally with distilled water in the increasing order of polarity using a Soxlet extractor. The yields of the concentrated crude extracts were estimated. There after crude extracts were subjected to preliminary phytochemical screening[20] and chemical tests were carried out; the results so obtained were tabulated. The herbal after collection from its venue was identified and authenticated by the taxonomist Dr S.K. Dash, Professor and Head, PG Department of Biosciences, CPS and the voucher herbarium specimens vide no. Ranjan / 08 /2008 and a live specimen for undertaking ex situ conservation and invitro studies of the specimen were deposited in the Museum of College of Pharmaceutical Sciences, Berhampur of Ganjam district, Odisha, for future reference.

2. Animal system used: Swiss albino mice (Mus musculus) weighing 20-30 gm and albino rats (Rattus norvegicus) of Wister strain weighing 160–200gm of either sex were used for the study. Animals were procured and maintained in animal house of College of Pharmaceutical Sciences (Mohuda) Berhampur, at least of 2 weeks prior to the study. For acclimatization the animal house was well maintained under standard conditions, at a temperature (22 ± 2^0C), room humidity ($60 \pm 10\%$) with 12 hours day and night cycle. Rats were housed in a group of 6 numbers per cage and mice in groups of 10 per cage. They were all provided with commercial food pellets and tap water ad libitum. Cleaning and sanitation was done daily, giving due importance. Paddy husk was provided as bedding material and was changed every day. All animal experiments were carried out as per CPSCEA committee of CPS, Mohuda: Regd. No. 1170/AC/08/CPCSEA.

3. Acute toxicity study: The tested extracts were administered orally. Fixed dose (c.f: OECD Guideline no. 423, Annexure 2d) method of CPCSEA was adopted for toxicity studies[21]. After subsequent administration of the drugs, animals were observed closely for first 2 hours for any toxic manifestations like motor activity, salivation, coma and death. Subsequent observations were made at regular intervals of 24 hours. Animals were under further observation up to a period of 4weeks [22, 23].

4. Medicament preparation: Two types of drug formulation of each were prepared i.e. 5% and 10% from each of the extracts, where 5gm and 10 gm of methanol and Chloroform extracts were incorporated with 100gm of simple ointment base BP [24].

5. Acute skin irritation study: 50 mg of each formulation of different concentration were applied over one sq cm area of intact and scrubbed skin of different animals. Aqueous solution of 0.8% formulation of the herbal extract was applied as standard irritant. The animals were observed for seven days for any sign of oedema and erythma [25, 26].

6. Wound healing studies: Wound healing property of Methanol and Chloroform extracts were studied on excision and incision wounds using Wister rats of either sex. Percentage of wound contraction and period of epithelization were measured in the excision wounds; whereas, tensile strength of healed wounds and total WBC count were measured in incision wounds.

Preparation of ointment from the test samples (herbal extract): Two different formulations of varied concentration were prepared by using an ointment base. Appropriate standard method of fusion was adopted, where the solid fats were melted, mixed and the triturate procedure was followed for preparation of the ointment. The methanol herbal (DQ) extract was incorporated in the ointment base to get two different concentrations (5 % and 10 %). All preparations were packed in wide mouth plastic jar with screw capped lid.

Neosporin (Welcome) and Supirocin (Glen mark) were used as standard ointments for control of microbes and expediting the healing process of the wound.

- **The Experimental Design:**

(a) Animals of either sex were divided into 11 groups of six animals each. Out of these, 7 groups were used for normal and 4 groups were used for diabetic models. Amongst the normal wound model: Group-I served as negative control (-ve) i.e. with distilled water; Group-II served as positive control (+ve) i.e. with simple ointment base; Group-III treated with a standard drug i.e. Neosporin ointment; Group-IV and V were applied with 5% and 10% of ointment based methanolic extract and Group-VI and VII with 5% and 10% ointment based Chloroform extract as test drug.

From the above observation, the selected effective drug extract was planned to be used as the test drug for ascertaining the comparative efficacy in diabetic models which was comprised of 4 groups. As usual, Group-I and II of the diabetic model served as the +ve and -ve control; Group-III stands for a standard ointment (Mupirocin) treated animal group and Group IV were treated with the effective test drug screened from the experimental findings of the normal wound model.

All groups of animals were applied topically with the respective ointments once daily.

(b) Appropriate standard method of fusion was adopted while preparing ointments from the test samples (DQ herbal extract). Neosporin (Welcome) and Supirocin (Glen mark) were used as standard ointments for control of microbes and expediting the healing process of the wound.

(c) Excision wound model: The animals were kept under starvation for 12 hours prior to wounding. The rats were inflicted with excision wound under light ether anesthesia [27-29]. Standard method was followed to create excision wound and Number of days required for falling of scab without any residual raw wound, gave the period of epithelization [30, 31]. Percentages of wound contractions were calculated on 4^{th}, 8^{th}, 12^{th} and 16^{th} days of post wounding period.

(d) Incision wound model: In incision wound model [32], two Para-vertebral straight incisions of 6cm each was made through the entire thickness of the skin on either side of the vertebral column with the help of sharp blade on anaesthetized animals. After complete homeostasis, the wounds were closed by means of interrupted sutures placed at equidistant points about 1cm apart, using 4-0 silk thread and curved needle No. 11[33]. Thereafter, extracts were applied continuously once daily for 10 days. Removal of suture was done on 8^{th} day of post wounding state and tensile strength of healed wound was measured on the 10^{th} post wounding day by continuous constant water flow technique of Lee [34]. Total WBC count was estimated on the very day of incision and on the 10^{th} day after incision [35, 36].

(e) Measurement of wound breaking strength of incised wounds: Measurement of wound breaking strength was performed following Lee's method with certain modifications [34].

(f) Induction of diabetes in the test organisms [37]: Diabetes was induced in rats by intra-peritoneal injection of Streptozotocin dissolved in 0.1 M sodium citrate buffer pH 4.5 at the dose of 50mg/kg body weight following standard treatises.

(g) Methodology for study of wound micro flora: A standard protocol [38] was followed, where plates, tubes and slides in triplicates, were prepared in each case for the purpose of isolation, screening ensuring identification with accuracy. The microbes; both aerobes and anaerobes, were isolated and cultivated using anaerobic jar and Pyrogallic acid - Sodium hydroxide method. They were identified based on agar slant cultural characteristics, Gram stain specificity and generalized specific biochemical characteristics.

(h) Statistical analysis: All results were expressed as mean±SEM. Significance of difference between control and drug treated groups were determined by one way ANOVA followed by students' t-test and the values $p \leq 0.01$ were being considered statistically significant.

III. Results

Phytochemical evaluation: Preliminary phytochemical analysis of the methanolic and chloroform extracts of the plant rhizome showed the presence of flavanoids, saponins, phenolic compounds, tannins, steroids carbohydrates etc. (Table 1). Individual detection tests for the phytochemicals were done following standard treatises for their identification [39].

Acute toxicity and skin irritation studies: It was found that the extracts did not show mortality at the dose of 2000 mg/kg body weight. Therefore, 2000 mg/kg dose was considered as ALD_{50} cut off the dose under Globally Harmonized Classification System (GHS) category 5 (safe dose), as per OECD guideline 423 (Annexure 2d). Common side effects such as, mild diarrhea, loss of weight and depression in treated group of animals were not recorded within 7 days of observation. In the acute skin irritation study, no sign of oedema and erythma were found during week days of observation too.

Wound healing studies: In excision wounds, the % closure of wound area for all tested extracts were found to increase significantly ($P \leq 0.01$) on 4^{th}, 8^{th}, 12^{th} and 16^{th} post wounding days in comparison with control (Tables

2&3 /Fig -1&2). As had been observed, the rate of wound contraction was significantly higher in the animals treated with methanol herbal (DQ) extracts compared to the reference drug i.e. Neosporin. Furthermore, the methanol herbal (DQ) extract exhibited significantly decreased period of epithelization compared to controls. Wounds dressed with both ME 10% & CH 10% found to be epithelized fastest (i.e. on 18 days); whereas, Neosporin dressed wounds epithelized on 20th day and the control group could be epithelized only up to 63% on 20th day. Similarly, the ME 5% and CH 5% dressed wounds epithelized little later (i.e. on 20th day) due to low concentration of the drug; but, it was significant from the point that doubling the dose was not yielding much healing efficacy. Though dose dependent healing was found with 10% extracts, 5% extracts were also with better efficacy, which worked at par with 10% extracts trialed with little increased concentration. The herbal (DQ) extract (ME 10%) showed significant wound healing activity comparatively better than that of commercial product of Neosporin (Table 2/Fig. 2); but, in diabetic induced rats Mupirocin / Supirocin worked better than the ME 10% test drug (Table 3/Fig.3). The results justified the wound healing properties of the plant in conformity with the folklore literatures, which could be comparable with Neosporin and Mupirocin/Supirocin respectively in normal and diabetic wounds to great extent. The tensile strength of the wound, undergone healing activity, was measured in all the groups; where, the 10% ME and 10% CH treated group showed increased tensile strength on 10th day as compared to all other groups (Table 4/ Fig. 3). Measuring another indicative parameter of healing, the total leukocyte count was performed; where in both the ME 10% and CH 10% test groups accounted comparatively less number of leucocytes than that of the control (Table 5/Fig. 4).

Microbial studies: No growth was observed after 5 days on the blood agar plates and thioglycolate tubes, as well. Smear prepared from the tube was '–ve' in standard groups and in the test groups, there was no infection of any microbe. However, samples taken on 4th, 8th and 12th day, marked growth were observed in the blood agar plates and thioglycolate tubes after 5 days of inoculation; thus, indicated severe infection and delayed healing of the wound particularly in the control groups and diabetes induced groups. Further, the presence of large Gram +ve rods and the production of the gas in the tubes suggested the presence of Clostridium species in the samples of diabetes rats. Anaerobic species were subjected to spore stain and other specified methods to discriminate amongst them. The Staphylococcus and streptococcus species were differentiated from each other by laboratory tests. Staphylococcus species were confirmed by tests like observing growth, fermentation, colonial pigmentation in mannitol salt agar media and also by DNase, haemolysis and novobiocin sensitivity tests. Similarly, Streptococcus species were confirmed by bacitracin test, haemolysis, CAMP test, bile esculin hydrolysis test and observing growth in media containing 6.5% NaCl at 10 ^{0}C and 45 ^{0}C. Multiple organisms like Streptococcus sp, Staphylococcus aureus, E. coli, B. subtilis, Klebsiella sp, Pseudomonas, Enterobactor sp, Enterococci sp, Clostridium sp, Candida sp etc were isolated from the wounds (Tables 6&7/Fig. 5&6). Aerobic and anaerobic microbial pathogens such as Propionibacterium sp., Clostridium sp. and Pseudomonas aeruginosa isolated as predominant bacterial species in diabetic wounds contrary to Pseudomonas aeruginosa and Staphylococcus aureus abode in the normal/induced wounds (Table 7/Fig. 5). Pattern and mode of isolation of microbial flora from normal and diabetic albino rats showed the diversity and polymicrobial nature of wound infection (Table 8/Fig.6).

IV. Discussion

'Wound healing', a process of universal recurring phenomenon in animal systems, comprises of different orderly phases [40-43], that restores cellular structures and tissue layers of the injured tissue intact. The tissue integrity is of questionable significance when microbes colonize, invade and proliferate. It is more pronounced when the tissue becomes ischemic, hypoxic or necrotic and the host immune response is compromised. Moreover, the microbial abundance and diversity greatly influences the fate of the wound that however, gets influenced by factors such as wound type, depth, location, quality and the level of tissue perfusion and the antimicrobial efficacy of the host immune response. Wounds may be either of acute or chronic type with varied etiology [44]. Acute wounds undergo healing within a time frame, predictable. In contrast, chronic wounds mostly caused by endogenous mechanisms that ultimately compromise the integrity of dermal tissue layers [45]. It may progress to an infected state due to polymicrobial [46-50] colonization involving many potential pathogens leading to systemic and local response like cellulites around a wound [51].

In our present investigation, wounds (both incision and excision type) were created on Swiss albino rats of Wister strain. Wound micro flora was screened from the excision model on respective days. Pure culture isolations of the organisms were tested for their identification and the same organism was taken as the test organism in the respective antimicrobial study against methanol extract used in the ointment base. Wound micro flora isolation and wound healing efficacy was also studied in the diabetes induced rats at parallel.

Phytochemical investigations of the extract showed the presence of triterpinoids, saponins, tannins and flavanoids. Several phyto-constituents like triterpinoids [52], saponin [53], alkaloids [54] and flavanoids [55] are known to promote wound healing process due to their antioxidant and antimicrobial activities. In addition triterpinoids reported to possess ability to increase the collagen content which is one of the factors promoting

wound healing [55]. Furthermore, healing activity is also attributed to free radical scavenging activity of flavanoids and triterpinoids. Both these class of phyto-constituents are known to reduce lipid per oxidation, not only by preventing or decreasing the rate of onset of cell necrosis; but, also by preventing vascularity. Lipid per oxidation is an important process in several types of injuries like burns, infected wounds, skin ulcers etc. so the drug that inhibit lipid per oxidation was also believed to increase the viability of collagen fibrils, which in turn resulted in increase in strength of collagen fibre by increasing the circulation, preventing cell damage and promoting DNA synthesis [56]. The result obtained in the experiment presented in the Table 3/Fig.3, showed an increase in the wound breaking and granuloma breaking strength on administration of the extract containing ointment.

Simultaneously, the excised rats were studied for microbial contamination and their impact on healing process particularly where quick and very slow healing was observed. The graphs and tables showed quantitative and qualitative wound infection and healing that often contain multiple organisms including aerobic and anaerobic gram +ve cocci and gram-ve bacilli and yeast like fungus Candida. The organisms like Streptococcus sp, Staphylococcus aureus, E. coli, B. subtilis, Klebsiella sp, Pseudomonas, Enterobacter sp, Enterococci sp, Clostridium sp, Candida sp etc were present as evident from the Table-6. Man is the most susceptibly vulnerable among all animals to Streptococcus infections. No organ or tissue of the body is completely immune to Streptococcus infection. So microbial contamination cannot be ignored and hence, their density/load was determined. The microbial load was found to be $> 10^4$ CFU/g of tissue in normal and $>10^7$ in dibetized wounds when observed in the Gram stained slides prepared from the sample. This quantification decreased in subsequent days of sampling which was indicative of the healing and antimicrobial property of the drugs. Still in some cases, healing was found to be hindered due to certain microbial flora. Aerobic pathogens such as S. aureus, P. aeruginosa and β-haemolytic streptococci were perhaps the organisms responsible for delay in healing. Staphylococcus aureus seemed to create obstacle as was isolated from slow healing group of rats and in the diabetes induced group of wounded rats. Literatures support that delay in healing of wound is due to S. aureus contamination in traumatic, surgical and burn wound infections and hence it is considered to be the most problematic pathogen associated with infected traumatic wounds [57, 58]. Poly microbial role of delayed healing has also been reported [46-50]. Isolation of β-hemolytic streptococci from diabetes induced wounded rats and from control groups were significant from the point that it perhaps stops closure of the wound though its load was not found to be high in the above groups. On application of the herbal ointment ME 10% on the diabetes induced group, comparatively better response was observed. However, the mupirocin ointment (standard) had better efficiency than the ME 10%. The test drug showed standard zone of inhibition in its individual antimicrobial sensitivity against streptococci that perhaps did not allow the growth of this microbe and hence the methanol and chloroform extract ointment groups (test group I, II, III and IV) had undergone timely closure of the wound which might be due to the action of the extracts on these organisms (Table 2/Fig. 2). Isolation of microbes in the wound samples taken from the 10% ME applied groups after 12^{th} day onwards showed the absence of S. aureus, P. aeruginosa and β-haemolytic Streptococci that might be resulting great reduction in WBC count, indicating the major role of the microbes in the healing process at the wound sites. As evident from the table-5, in incision wound models, the WBC count reduced from 7939±345.7 to 5614±83.2 (cells/mm^3) and 8399±244.6 to 5482±83.3 (cells/mm^3) in test II and test IV groups respectively. The wound closure was observed at a greater rate in these groups from 294±11.5 on 8^{th} day to 107±7.4 on 12^{th} day and subsequently to 25±3.2 on 16^{th} day and finally closed on the 18th day in test group II. Similarly, in test group IV, wound closure increased from 290±12.6 on 8^{th} day to 104±6.3 on 12^{th} day and subsequently, to 27±3.9 on 16^{th} day; thereby resulting a total closure on 18th day were observed. Further, it was noticed that where the number and type of organisms found at high frequency, the pathogenecity and severity of infection were also very high there, compared to the control groups. This might be due to many reasons such as:

1. Nutrients produced by one type of microbe may help in growth of others.
2. Virulence factors produced by the established pathogen might render advantage to other associated microbes.
3. Consumption of oxygen by aerobic flora in wounds favours the growth of anaerobes.
4. These anaerobes might be creating hindrances in proper functioning of the immune cells; thereby, taking advantage of their own growth along with other cohabitants.

Wounds created or formed in diabetic patients; whose probability of infection is five times greater than in non diabetic patients; because, neutrophil, chemotaxis, phagocytosis, intra cellular killing mechanisms, serum opsonic activity are impaired in diabetic patients [59, 60]. High level of glucose in wound exudates stimulates the production of succinate in gram negative bacteria that can impair host cell function and the host becomes more susceptible to infection. Microbial contaminated wounds undergo delay in healing and closure or sometimes impaired due to exo- and endo- toxins, adhesion factors like virulence factors produced by the pathogens. Adhesion factors such as fimbri (pilli), M-proteins, opa-proteins, lipoteichoic acid, haemaglutinins, extracellular proteins and enzymes such as hyaluronidase, streptokinase, proteases, collagenase, leucocidins, porins,

fibrinolysin, gelatinase, elastage, DNase, K-toxins etc and exotoxins such as AB exotoxins, membrane disrupting, super antigen exotoxins etc and endotoxins like LPS (Lipopolysaccharide) and antiphagocytic factors like capsules, short chain fatty acids etc, directly or indirectly can be a factor for impairing of the wound healing process [61].

In the course of investigation, diabetic rats were taken as a system parameter in which efficacy of the drug prepared from the herbal extract was tested. It was found that Streptozotocin induced diabetic rats were also observed to be undergoing healing equally as that of the -ve control group. This diabetic group of rats was under medication of ME 10% ointment which was responding positively that might be due to keeping the cellular mechanisms and other required factors of the host intact like up regulation of collagen expression; angiogenesis [62, 63]; increase in tensile strength [64] etc improves circulation providing oxygen and nutrient required for healing process [65].

V. Conclusion:

There has been constant endeavour to ascertain drugs from the herbal world combating against wounds of varied nature. The above work enriched the medico folk lore data base pertains to scientific validation of such therapeutic potency of Drynaria quercifolia Linn. at par.

Competing Interest:

The authors declare that there is no conflict of opinion on any issue pertains to the paper and have no financial / non financial competing interests in any respect.

Acknowledgements:

Thanks are due to the authorities of PGDBS, CPS, Berhampur (at: Mohuda) for providing facilities and to the Berhampur University (BU) in approving of the Ph. D. project for registration of one of us (Ranjan Padhy) under supervision of Prof. S.K. Dash. Thanks too, to Prof. (Dr) Balabhadra Tripathy, Head, P.G. Dept. of English, BU for going through the script for rectifying the script with necessary throughput expression of the facts.

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Table 1: Preliminary phytochemical test results of methanolic and chloroform extracts obtained from rhizome of **Drynaria quercifolia** Linn.

Test for the phytochemicals	Methanolic extract of the rhizome	Chloroform extract of the rhizome
Alkaloids	-ve	-ve
Flavonoids	+ve	+ve
Phenols	+ve	+ve
Proteins and Amino acids	-ve	-ve
Saponins	+ve	-ve
Tannins	+ve	+ve
Steroids	+ve	+ve
Carbohydrates	-ve	+ve

'+ve' and '–ve' indicates presence and absence of the phytochemicals in the extract respectively.

Table-2: Topical application of ointments prepared from extracts of DQ on excision wound Model % of wound healing.

Group ↓	Post Wounding Days →						
	0 Day	4th Day	8th Day	12th Day	16th Day	18th Day	20th Day
Control (negative)St.dist. H_2o	619 ±32.5 (0.0)	543 ±20.7 (12.27)	450 ±10.8 (27.30)	369±15.2 (40.38)	284±4.6 (54.11)	250±0.8 (59.11)	228±0.5 (63.16)
Control (positive) Oint. base	619±32.5 (0.0)	543 ±20.7 (12.27)	459 ±16.8 (25.84)	359±10.2 (40.38)	276±8.6 (55.41)	252±5.8 (59.28)	224±4.2 (63.81)
Standard Neosporin	616±35.8 (0.0)	411±27.4* (24.30)	281±15.2* (38.77)	163±4.0** (55.82)	30±0.8** (89.13)	20±0.5** (92.06)	0.00** (100)
Test – I (ME 5%)	614±25.7 (0.0)	465± 17.5 (14.36)	342±12.7** (25.49)	293±5.8** (20.59)	45±1.6** (83.69)	22± 0.8** (91.26)	0.00** (100)
Test – II (ME10%)	611±29.3 (0.0)	442±22.3* (18.60)	294±11.5** (35.94)	107±7.4** (71.00)	25±3.2** (90.94)	0.00** (100)	--------
Test – III (CH 5%)	612±24.1 (0.0)	467± 16.4 (13.99)	344±13.8* (20.05)	295±7.9** (20.05)	47±1.8** (82.97)	23± 0.8** 90.87	2.12±0.2** (99.05)
Test – IV (CH 10%)	610±27.2 (0.0)	435±21.2* (19.88)	290±12.6** (36.81)	104±6.3** (71.81)	27±3.9** (90.21)	0.00** (100)	----

Results are expressed as mean±SEM of six readings; significance evaluated by one way analysis of variance (ANOVA) followed by student t-test versus control group *p<0.01, **p<0.001. Figures in parenthesis indicate the percentage of wound healing. The ointment formulations of different concentrations are comparable with control and standard.

Table – 3: Topical application of ointments prepared from extracts of DQ on excision wound model of diabetic rats

[% of wound healing = (1- t/c) X 100]

Group ↓	Post Wounding Days →						
	0 Day	5th Day	10th Day	15th Day	20th Day	25th Day	30th Day
Control (negative)	611±29.3 (0.0)	543 ±20.7 (11.12)	459 ±15.8 (24.87)	359±10.2 (41.24)	276±8.6 (54.82)	252±5.8 (58.75)	224±4.2 (63.33)
Control (positive)	619±30.5 (0.0)	540 ±10.5 (12.76)	467± 16.4 (24.55)	389 ±15.8 (37.15)	281±4.4 (54.60)	262±5.6 (57.76)	214±5.3 (65.42)
Standard Mupirocin	616±35.8 (0.0)	381±15.2* (29.44)	248±0.8** (46.89)	108±0.5** (72.23)	56 ± 4.5** (80.07)	06±0.5 (97.70)	2.0 ± 0.2 (99.06)
Test drug (ME-10%)	614±25.7 (0.0)	342±12.7* (36.66)	274±10.4* (41.32)	176 ±7.9** (54.75)	95 ±1.8** (66.19)	45 ±0.8** (82.82)	5.12±0.2** (97.60)

Results are expressed as mean±SEM of six readings; significance evaluated by one way analysis of variance (ANOVA) followed by student t -test versus control group *p<0.01, **p <0.001. Figures in parenthesis indicate the percentage of wound healing. The ointment formulations of different concentrations are comparable with control and standard.

Table-4: Topical application of ointment based extracts prepared from of Drynaria quercifolia on incision wound model as on 10th day.

Group	Treatment	Tensile Strength in Grams±SEM of healed wounds
Control	Simple ointment base	530 ± 12.5
Standard	Neosporin ointment	650 ± 15.7*
Test- I	5% methanol extract	630 ± 11.1*
Test- II	10% methanol extract	675 ± 10.5*
Test- III	5% chloroform extract	638 ± 12.7*
Test -IV	10% chloroform extract	665 ± 13.8*

Results are expressed as mean±SEM of six readings of six animals of each group. Significance evaluated by one way analysis of variance (ANOVA) followed by student t-test.

Table-5: Total leukocyte count on 0 day and on 10th day (Incision Wound)

Group	WBC count (Expressed in no. of cells/mm^3)	
	0 Day	10th Day
Control	8780 ± 519.3	7691 ± 659.1
Standard	8422 ± 346.2	6695 ± 378.4
Test – I [5% (ME)]	8120 ± 375.8	5735 ± 133.7*
Test – II [10%(ME)]	7939 ± 345.7	5614 ± 83.2*
Test – III [5% (CH)]	8122 ± 365.7	5514 ± 125.5*
Test – IV [10% (CH)]	8399 ± 244.6	5482 ± 83.3*

Results are expressed as mean±SEM of six readings of six animals from each group. Significance evaluated by one way analysis of variance (ANOVA) followed by student t- test.

Table 6(a): Identification of wound microflora (cultural characteristics and Staining Techniques)

Pure culture isolate No.	Gram stain characters (bacterial)/ Lacto phenol cotton blue stain (fungal)	Agar slant cultural characters(bacteria)/ Sabouraud agar plate characters(fungi)
Pure culture isolates of aerobically grown sample of the excision wound		
1	Rod , - ve	White , moist, glistening growth
2	Coccus, +ve	Abundant, opaque, white waxy growth
3	Coccus, + ve	Abundant, opaque, golden yellow growth
4	Rod , + ve	Club shaped, luxurious growth in Loffler's serum medium
5	Rod, - ve	Slimy ,white, somewhat translucent raised growth
6	Rod , -ve	Thin ,white medium turns green
7	Coccus, +ve	Abundant, opaque, yellowish growth
8	Coccus, +ve	Thin, even growth
9	Yeast like fungus, produces pseudo mycelium	Colonies are small, round, colourless, moist with unbroken even edges
10	Coccus, +ve	Thin, even growth
11	Coccus, +ve	Abundant, opaque, golden yellow growth
Pure culture isolates of anaerobically grown sample from the excision wound		
12	Coccus, +ve	Thin , even growth (pyrogallic acid crystal agar slant)
13	Rod, + ve	Abundant, white growth(pyrogallic acid crystal agar slant), gas producers (thyoglycolate tube), spore forming(Schaeffer Fulton method)
14	Rod , + ve	Abundant, white growth (pyrogallic acid crystal agar slant), non spore forming (Schaeffer Fulton method).
15	Rod, - ve	Abundant, pigmented growth

Table 6(b): Identification of wound micro flora (By biochemical characterization).

Test	Pure Culture Isolate Nos.										
	Isolate 1	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**	**Isolate 6**	**Isolate 7**	**Isolate 8**	**Isolate 9**	**Isolate 10**	**Isolate 11**
Indole	+ ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-	-ve	-ve
Methyl red	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-	+ve	+ve
Voges proskauer	-ve	+ve	±	±	±	-ve	±	±	-	±	±
Citrate utilization	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	-	-ve	-ve
Gas production	AG	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-	+ve	-ve
H_2S production	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-	+ve	-ve
Catalase activity	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve
Oxidase activity	-ve	+ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
Urease activity	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	-	-ve	-ve
NO_3 reductase	+ve	+ve	+ve	±	+ve	+ve	+ve	+ve	-	-ve	+ve
Carbohydrate utilization test	AG	-ve	A	-ve	AG	-ve	A	A	-	A	A
Results	**E. coli**	**Bacillus subtilis**	**Staphylococcus sp.**	**Corynebacterium sp.**	**Klebisiella pneumonia**	**Pseudomonas aeruginosa**	**Staphylococcus sp.**	**Streptococci sp.**	**Candida sp.**	**Streptococcus sp.**	**Staphylococcus sp.**

Table 6(c): Tests for identification of Staphylococcus and Streptococcus micro flora.

Specialized tests	**sample from staphylococcus colony**			**Specialized tests**	**Sample from streptococcal colony**	
Mannitol salt agar						
Growth	+	+	+	Hemolysis	β	β
Fermentation	-	+	-	Bacitracin test	+	-
Colonial pigmentation	white	yellow	white	CAMP test	-	+
Coagulase	-	+	-	Bile esculin test	-	-
DNase	-	+	-	6.5%NaCl medium	No growth	No Growth
Haemolysis	-	β	-	Growth at 10^0 C	No Growth	No Growth
Novobiocin Sensitivity	sensitive	sensitive	resistant	Growth at 45^0 C	No Growth	No Growth
Result	**S. epidermidis**	**S. aureus**	**S. saprophyticus**	**Result**	**S. pyogenes**	**β hemolytic Streptococci**

Table 7: Aerobic and Anaerobic bacterial isolates from acute induced wounds of diabetic and normal albino rats**.**

Name and type of bacterial isolates	**Samples from normal rats (total 18 samples)**		**Samples from diabetic rats (total 18 samples)**	
Aerobic bacteria	**Frequency of cases**	**Percentage (%)**	**Frequency of cases**	**Percentage (%)**
E. coli	7	10.76	5	6.41
Bacillus subtilis	0	--	2	2.56
Staphylococcus aureus	8	12.30	7	8.97
Corynebacterium sp.	7	10.76	2	2.56
Klebsiella pneumonia	4	6.15	4	5.12
Pseudomonas aeruginosa	16	24.61	12	15.38
Staphylococcus epidermidis	7	10.76	5	6.41
β hemolytic Streptococci	3	4.61	0	--
Candida sp.	1	1.53	1	1.28
Streptococcus pyogenes	2	3.07	8	10.25
Staphylococcus saprophyticus	4	6.15	2	2.56
Anaerobic bacteria	**Frequency of cases**	**Percentage (%)**	**Frequency of cases**	**Percentage (%)**
Peptostreptococcus sp.	1	1.53	2	2.56
Clostridium sp.	0	-	8	10.25
Propionibacterium sp.	4	6.15	18	23.07
Gram-ve pigmented bacillus	1	1.53	2	2.56

Table 8: Pattern of isolation of wound micro flora from wounds of diabetic and non-diabetic albino rats.

Pattern or mode of isolation of bacteria	**Normal rats**		**Diabetic rats**	
	Frequency/ No. of cases	**Percentage (%)**	**Frequency/ No. of cases**	**Percentage (%)**
Single pathogen	2	11.1	3	16.6
Double pathogen	8	44.4	3	16.6
Three pathogens	5	27.7	7	38
More than three pathogens	3	16.6	5	27
Total	**18**		**18**	

Figure-1: Percentage of healing in different groups on different days in normal rats. Test II (ME 10%) was found to be the best amongst all.

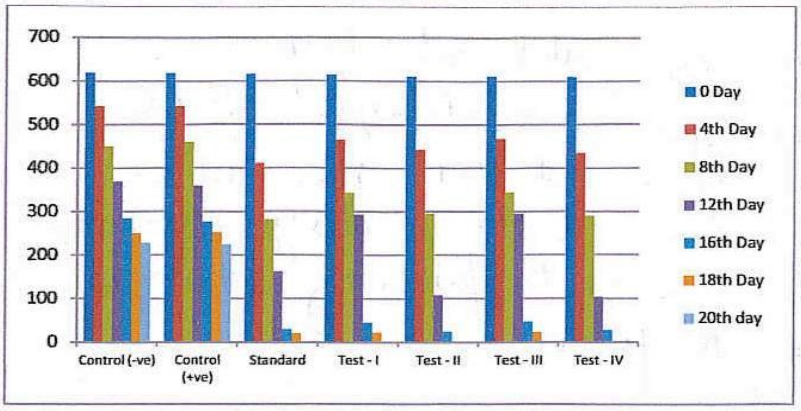

Figure-2: Percentage of healing in different groups on different days in diabetic rats. Standard is more efficient than the Test II (ME 10%)

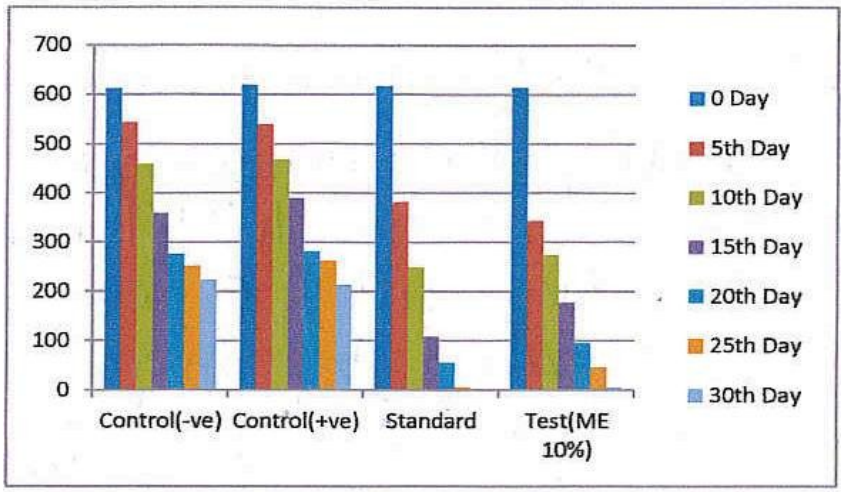

Figure -3: Measurement of tensile strength (in gm) in incision models of normal rats on 10th day. Test II (ME 10%) applied group showed better tensile strength than standard,

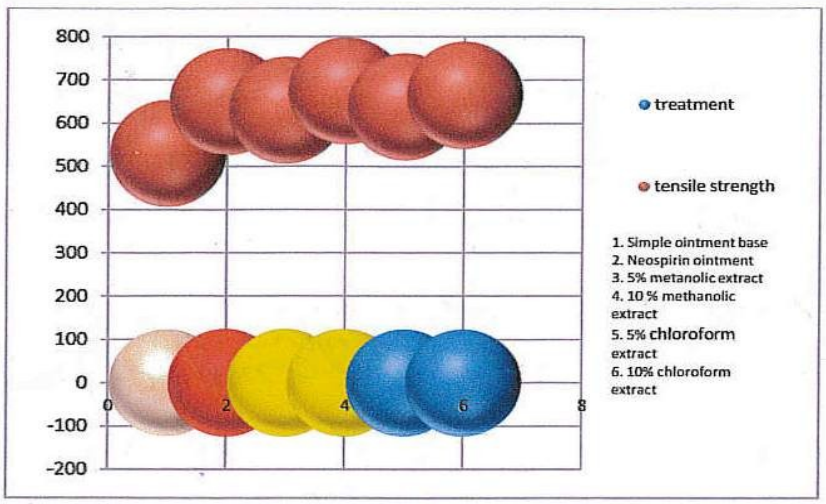

Figure-4: Total leukocyte count decreased significantly in Test II (ME 10%) applied group as compared to standard.