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ANTIBACTERIAL, ANTIOXIDANT, ANTIMICROBIAL AND WOUND HEALING POTENTIAL OF TRITICUM AESTIVUM & TERMINALIA BELLIRICA

Yashpal Sangwan1, Tanuj Hooda2, Ramchander Khatri2

1. Nav Chetna Institute for Pharmacy Professionals

2. Vaish Institute of Pharmaceutical Education and Research, Rohtak, Haryana

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Corresponding Author-
Yashpal sangwan
H.NO. 450 Urban Estate Near Jaat Dharamshal Jind-126102, Haryana, India
E-mail:
sangwan.yashpal@gmail.com
Mobile No.: +91-9812384235

ABSTRACT:
Objective: To investigate the ethanolic and aqueous extracts of Terminalia bellirica, Triticum Aestivum for potential of antioxidant, antibacterial, antimicrobial and wound healing activity. Methods: The antioxidant, antibacterial, antimicrobial activity of extract was studied against the 8 bacterial and 2 fungal strains using agar cup plate method. Antibacterial activities were evaluated against five microorganisms using agar well diffusion method the effect of 5% and 10% ointments of Terminalia bellirica, Triticum Aestivum ointments were evaluated by incision and excision model in rats. The wound healing activity of 5% & 10% ointment were assessed by the breaking strength, % wound contraction and period of Epithelialization. Results: Antibacterial activities were evaluated against two microorganisms in which T. Billerica; T. Aestivum showed significant activity with a MIC of 1.562 mg/ml, 3.125 mg/ml respectively. The plant extracts of brown raisins T. bellirica, T. Aestivum showed remarkable antioxidant activity. The results showed that both the ointments exhibited significant antimicrobial activity against all the tested microorganisms and have significant wound healing activity as evident from the breaking strength; % wound contraction and period of epithelialization. The topical application of individual and combination of plant extracts on wounds caused significantly faster healing in wound area as compared to the commercial ointment. Conclusions: The present study provides a scientific rationale for the traditional use of these plants in the management of wounds. These results shows that the combination of these plants extracts possess effective wound healing properties due to their antimicrobial and antioxidant activities by possessing the active compounds.

Introduction:

Herbal drugs play a role in the management of various disorders; most of them speed up the natural healing process of humans. Herbal medicines have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha. Medicinal components from plants play an important role in conventional as well as western medicine. Numerous medicinal plants and their formulations are used for various disorders in ethno medical practices as well as traditional system of medicine in India. Mostly herbs contain polyphenols which are most powerful natural antioxidants and are highly valued for their antioxidant and anti-aging effects. Antioxidants are widely used as ingredients in dietary supplements and are exploited to maintain health and prevent oxidative stress- mediated diseases. Antioxidant compounds like phenolic acids, polyphenols and flavonoids inhibit the mechanism that leads to degenerative diseases. A wound may be defined by as a break in the epithelial integrity of the skin or may also be defined as a loss or breaking of cellular and anatomic or functional continuity of living tissue. Wound healing studies are mainly aim to detect various means and factor influencing healing process, so they could be either used or avoid in clinical practice to favorably alter the healing process as stated. Review of literature revealed that the wound healing property of this plant has not been subjected to scientific evaluation and there is not enough scientific data to support the claims made in the ancient literature. The need for safer and effective wound healing drug and the lack of enough scientific data to support the claims made in ancient literature prompted the present study.

Materials and Methods

Collection and authentication of plant material

Plants were collected from road side between Shimla and Solan (Himachal Pradesh) and from Coimbatore district Tamil Nadu. The collection was done in the month of August. It has been authenticated by Prakash Singh, Reader, Dept. of Botany, A.N, College, Patna, and Bihar, India. The specimen receipt was submitted in the department for future reference.

Preparation of the leaves extract

Ethanolic extract of Terminalia bellirica, Triticum Aestivum leaves

The leaves were removed from plant and dried in shade then were powdered mechanically to get the coarse powder. Weighed quantity of coarse powder (1 kg) was extracted with ethanol at 40-50 o C up to 22 hrs. in soxhlet apparatus. A brown semi – solid extract was obtained after concentrating ethanolic extract. Then the extract was subjected to phytochemical analysis and checked for antioxidant and antimicrobi activities

Ointment formulation

The extracts were used as ingredient for 5% & 10% ointment preparation. About 5 g & 10 g of semisolid extract were incorporated into the 100 g of simple ointment base B.P. Simple ointment base was used as control group. Extract ointment was used twice daily to treat different groups of animals and povidone iodine ointment used for the standard group.

Phytochemical screening

Preliminary phytochemical screening of the ethanolic extract of Terminalia bellirica, Triticum Aestivum leaves was performed to test the presence of the active chemical constituents.

Antioxidant activity

DPPH radical scavenging assay

The procedure was followed by the method of Sanchez- Moreno et al and Narendhirakannan et al.

Hydrogen peroxide scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al.

Assay of Reducing Power

The total reducing power of the extracts was determined according to the method of Chang et al and Tevfik et al.

Antimicrobial activity

For antimicrobial activity 4 Gm + ve, 4 Gm-ve and 2 fungal strains are used.

Gram-positive bacteria: Bacillus subtillis, Staphylococcus aureus, Bacillus Cereus, Micrococcus luteus.

Gram-negative bacteria: Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi Fungi: Aspergillus niger, Candida albicans.

Minimum Inhibitory Concentration (Zone of Inhibition)

Agar Cup Plate Diffusion Method.

For Gm+ve and Gm-ve Bacteria: Agar Media is used.

For Fungal Strain: Saboraud dextrose agar media is used.

Preparation of the tested organisms

A) Preparation of standard bacterial suspensions: The average number of viable Bacillus subtillis, Staphylococcus aureus, Bacillus Cereus, Micrococcus luteus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi organisms per ml of the stock suspensions was determined by means of the surface viable counting technique. About (108 - 109) colony-forming units per ml was used. Each time, a fresh stock suspension was prepared; the experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

B) Preparation of standard fungal suspensions: The fungal cultures Aspergillus niger, Candida albicans were maintained on Saboraud dextrose agar, incubated at 25ºC for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in (100ml) of sterile normal saline and the suspension was stored in refrigerator till used.

In vitro testing of extracts for antimicrobial activity:

Testing for antibacterial activity:

activity of the prepared extracts. 0.6 ml of standardized bacterial stock suspensions (10 -10) colony- forming units per ml was thoroughly mixed with 60 ml of sterile nutrient agar. 20 ml of the inoculated nutrient agar were distributed into sterile Petri dishes. The agar was left to set and in each of these plates 4 cups, 10 mm in diameter, were cut using a sterile cork borer no. 4 and the agar discs were removed. Alternate cups were filled with 0.1ml of each extracts using micro titer-pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37ºC for 18 hours. Two replicates were carried out for each extract against each of the test organism. Simultaneously addition of the respective solvents instead of extracts was carried out as controls. After incubation the diameters of the results and growth inhibition zones were measured, averaged and the mean values were tabulated.

Testing for anti-fungal activity:

The same method as for bacteria was adopted. Instead of nutrient agar, yeast and Mould extract agar was used. The inoculated medium was incubated at 25ºC for two days for the Candida albicans and three days for Aspergillus niger.

Wound healing activity Animals

Wistar albino rats of either sex weighing between 180 and 200 g were obtained from Nitin Biologicals, New Delhi, India the study was approved by the Institutional Ethics Committee for animal experimentation PDM School of Pharmacy, Safidon (PDM/IEAC/12/08/04), Safidon, Haryana (India) and all the procedures on animals were carried out as per CPCSEA guidelines, India. The animals were acclimatized to standard laboratory conditions of temperature (22±30C) and maintained on 12:12 h light: dark cycle. They were provided with regular rat chow (VRK laboratory animal feed).

Acute skin irritation test

The study was carried out as suggested by on rats. An area measuring about 500 mm2 on the dorsal fur of the animals was shaved. The prepared ointments were applied separately to different groups of animals. After 4 h, the skins were observed for signs of inflammation.

Excision wound model

Excision wounds were created by excising a circular piece (500 mm2 in area) of full thickness skin from the dorsal interscapular region. Wound contraction was monitored by measuring wound area, planimetrically, on alternate days till the wounds were completely healed. This was expressed as percentage of wound contraction. Time taken for complete epithelialization was noted by recording the days required for fall of scab leaving no raw wound behind.

Incision wound model

All animals were anaesthetized before wound creation and two paravertebral long incisions were made through the skin at the distance of about 1.5 cm from midline on each side of the depilated back of rat. The both edges kept together and stitched with black silk surgical thread (no. 000) and a curved needle (no. 11). The continuous threads on both wound edges were tightened for good closure of the wound. After stitching, extract and dexamethasone were applied daily and on alternate days respectively up to 9 days; when wounds were cured thoroughly the sutures were removed on the day 9 and tensile strength of cured wound skin was measured using tensiometer.

Antibacterial activity

The antibacterial activity was tested using agar well diffusion and broth dilution methods according to Arshad H and Lino A, et al. The MTCC cultures were obtained from kovai medical centre, Coimbatore, Tamil Nadu. All the plant extracts were tested against Staphylococcus aureus, Klebsiella pneumoniae, Enterococcus faecalis, Escherichia coli and Pseudomonas aeroginosa.

Agar well diffusion method

The antimicrobial activity was tested against (ethanol) leaves of Azadirachta indica, Emblica officinalis, Terminalia bellirica, Terminalia chebula (Triphala), Cleome gynandra, Curcuma longa, Triticum aestivum, Vitis vinifera L – Black Raisins (Zante currants) and brown raisins (Sultanas). 1 ml of the test culture (107 CFU/ml) was inoculated into a sterile plate with 20 ml Muller Hinton molten agar which was made to solidify. 5 wells of approximately 6 mm in diameter were made on the surface of the agar plates using a sterile borer. Stock solution of each plant extract was prepared at concentration of 50 mg/ml in ethanol. Each well was filled with 0.10 ml of the plant extracts. 0.10 ml of ethanol was taken as negative control and 10 micro g of streptomycin served as a positive control respectively. The plates were then incubated at 37 0C for 24 h and zone of inhibition was measured. The results were then tabulated.

Statistical analysis

The data obtained by the various parameters was statistically evaluated by one way analysis of variance (ANOVA) followed by dunnet t test using Graph Pad Prism software (Graph Pad software Inc., Version 4.0.0.255). The mean values ± SEM were calculated for each parameter. The differences and the changes in healing by the plant extracts treated groups against the control group and standard group were analyzed accordingly. Level of significance was kept at $P<0.05$.

Results

Yield of plant extract

The percentage yield of the dried ethanolic and aqueous extracts of V. vitigenia roots was found to be 8.58% and 1.73% respectively.

Phytochemical screening of Terminalia bellirica,

Triticum Aestivum

Preliminary phytochemical screening of the etanolic and aqueous extracts showed the presence of terpenoids, saponins, tannins and flavonoids.

Antimicrobial activity

Table 1: Antimicrobial activities of 10 mg/ ml of *Terminalia bellirica, Triticum Aestivum* extract (Minimum Inhibitory Concentration)

Sr No.	Strain	Organism	Standard	Alcoholic	Aqueous
			Zone of inhibition(mm)		
1	Gram positive	*Bacillus subtilis*	23	15	17
2	Gram positive	*Staphylococcus aureus*	21	14	16
3	Gram positive	*Bacillus Cereus*	30	14	16
4	Gram positive	*Micrococcus luteus*	31	**21**	**22**
5	Gram negative	*Escherichia coli*	30	**13**	**21**
6	Gram negative	*Proteus vulgaris*	32	**20**	**23**
7	Gram negative	*Pseudomonas aeruginosa*	30	**19**	**22**
8	Gram negative	*Salmonella typhi*	23	15	16
9	Fungal	*Aspergillus niger*	30	**18**	**23**
10	Fungal	*Candida albicans*	20	17	18

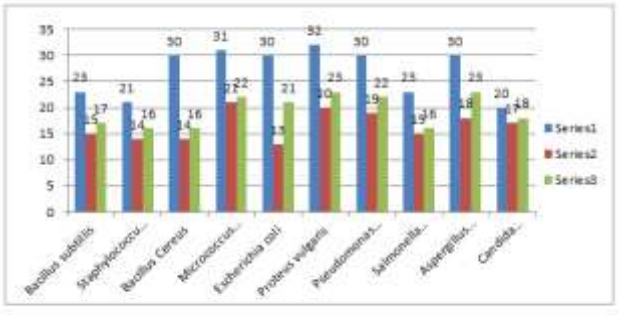

Figure 1: Minimum inhibitory concentration

For bacterial strain Gentamicine 50 µg/ml and for fungal strain Amphoteracine B 50 µg/ml is used. Both the plant extracts show good antimicrobial activity against Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris,

Aspergillus niger strains. Proteus vulgaris mainly cause the wound infection and these both the extracts show good antimicrobial activity. It is seen that aqueous extract shows good activity as compared to that of alcoholic extract. Both the activity shows better activity in gm-ve bacteria.

Wound healing study

Acute skin irritation test

In the skin irritation study, the tested ointment did not show any type of irritation and there was no evidence of any noticeable inflammation on the skin.

Wound contraction

The percentage wound contraction was determined using the following formula:

Percent wound contraction = Healed area / Total wound area × 100

Wound area was measured by tracing the wound margin using a transparent paper in each 2 days interval and healed area calculated by subtracting from the original wound area. On day 4, the wound contraction of standard and extract ointment treated groups was found to be significant ($P < 0.05$) in comparison to simple ointment base treated group. On day 13, 5% aqueous extract ointment treated wound was completely healed while 5% alcoholic extract ointment treated group was also almost at complete healing stage. On day 18, standard ointment treated group healed 100% and simple ointment base treated group showed 95.71% healing. It was also observed that epithelialization period of treated and standard group were less in comparison to simple ointment base treated group Results are shown in Table 2 and Table 3

Table 2: % Wound Contraction (Mean ± SEM)

Group	% Wound Contraction On 13th day (Mean ±Sem)
Control group	64.7 ± 1.2
Standard group	75.45** ± 1.76
10% ointment	**96.03*** ± 1.95**
5% ointment	**95.08 ***± 1.35**

(n=6, ***= Aq. versus Control (P< 0.001), ***= Alc. versus Control (P<0.001), **= Standard versus Control (P<0.001)

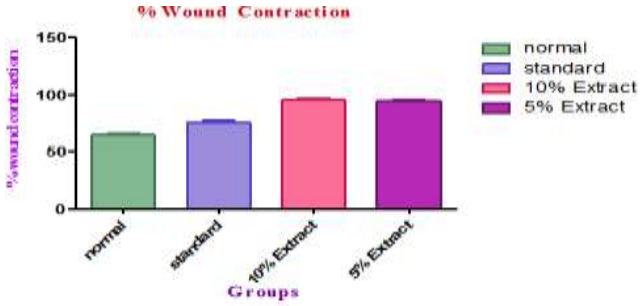

Figure 2: % Wound contraction in Excision Wound Model

Table 3: Period of Epithelialization (Mean ± SEM)

Groups	Period of Epithelialization (Mean ±SEM) (In Days)
Control group	23.33±0.33
Standard group	19.66**±0.33
10% ointment	15.33***±0.33
5% ointment	13.66***±0.33

(n=6, ***= Aq. versus Control (P< 0.001), ***= Alc.versus Control (P<0.001), **= Standard versus Control (P<0.001)

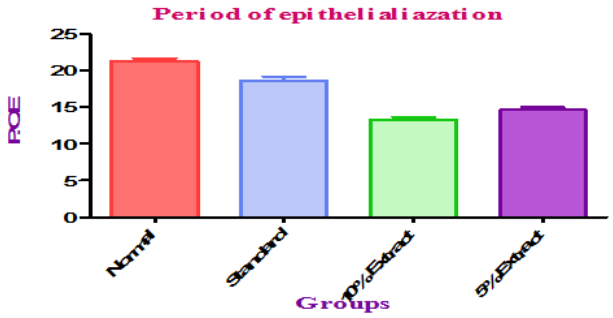

Figure 3: Period of Epithelialization in Excision Wound Model

Tensile strength of incision wound model

The breaking strength of the incision wounds was increased in drug treated groups to significant extent, i.e., 586.66 ***± 1.66 in aqueous extract, and 521.66 ***± 3.33 in alcoholic extract ointment treated group as compared to control group. The results are also comparable to standard drug Povidone iodine ointment. Tensile strength for the treated group on 10th day was found to be very significant ($P < 0.001$) than control group as shown in Table 4.

Table 4: Breaking Strength (Mean± SEM)

Groups	Breaking strength(Mean ±SEM)(kg/cm²)
Control group	258.33 ± 1.66
Standard group	361.66**± 1.66
5% ointment of Aqueous extract	588.66 ***± 1.66
5% ointment of Alcoholic extract	525.66 ***± 3.33

(n=6, ***= Aq. versus Control (P< 0.001), ***= Alc. versus Control (P<0.001), **= Standard versus Control (P<0.001).

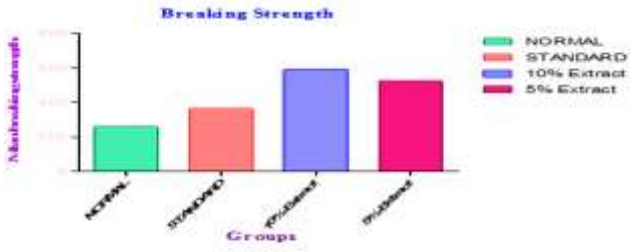

Figure 4: Breaking Strength in Incision Wound Model

In incision wound model on 10th day, 5% Ointment of aqueous extract and alcoholic extract shows good result in comparison to standard and normal group.

Discussion

Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and herbivores. Showed the presence of various phytochemicals which can be attributed to have antioxidant, antibacterial and wound healing properties. The test showed the presence of nearly all the polyphenols that were tested. Alkaloids, flavonoids, tannins, saponins, anthroquinones, phlobatannins, triterpenoids, lipids/fat. The plant shows the presence of tannins, saponins and flavonoids so this antimicrobial activity may be due to these phytoconstituents. This may therefore explain the demonstration of antimicrobial activity by the root extracts of Terminalia bellirica, Triticum Aestivum. The demonstration of antibacterial activity against both gram positive and gram negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds.

Wound healing, a complex sequence of events, is initiated by the stimulus of injury to the tissues. A positive stimulus may result from the release of some factors by wounding of tissues. Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue. From the above result of excision wound model it is evident that on the day 13th day, there was significant increase in wound contraction in both the groups compared to control groups. The tensile strength with incision model showed maximum activity for wound healing and the result was significant (P< 0.01), i.e. 588.66 ***± 1.66 with 5% aqueous extract and 525.66

***± 3.33 in comparisons to control. In Terminalia bellirica, Triticum Aestivum extract shows the presence of phytoconstituents like tannins, saponins and flavonoids are known to promote wound healing process. The study reveals that 5% extracts treated groups possesses good wound healing properties which may be attributed to the individual or combined action of phytoconstituents like tannins, saponins and flavanoids present in it. It can also be concluded that the maximum wound healing exerted by the aqueous extract of Terminalia bellirica, Triticum Aestivum.

Conclusion

This investigation has opened up the possibility of the use of this plant in drug development for human consumption possibly for the treatment of gastrointestinal, urinary tract and wound infections and typhoid fever .In conclusion, the results of study showed that the aqueous and alcoholic extract ointment of Terminalia bellirica, Triticum Aestivum effectively stimulates wound contraction in excision wound model and increase tensile strength of incision wounds as compared to control group and standard group. These finding could justify the inclusion of this plant in the management of wound healing.

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