FORMULATION OF OINTMENT USING GREEN TEA AND FISH SCALE CHITOSAN FOR WOUND HEALING ACTIVITY

Abstract

Wound healing is a complicated process, microbes residing in them make it worser and delay the process. Commercial ointments are being the only source in practice. There are plenty of natural remedies at our sight. Among those green tea and chitosan extracted from fish scales was promising sources. S.aureus, P.aeruginosa and E.coli were isolated from the wound sample. The catechin compounds EGCG, ECG and chitosan, cationic polymer attributed antimicrobial activity through well-diffusion method. At 50µl concentration of chitosan the highest zone of inhibition was 16.3mm (E.coli), ethanolic extract of green tea 50 µl showed maximum 18.4mm zone of inhibition for S.aureus. gel was formulated at specific concentrations. The FTIR analysis of . It is said that the combination of green tea extract and chitosan together plays a significant role in inhibiting the wound pathogens and aids in quick healing.

Keywords: Chitosan; green tea; wound pathogens.; Gel formulation; wound healing

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I. INTRODUCTION

Body's immediate line of defense against assailant is the integumentory layer. The nature of the skin can be damaged by tears, cuts trauma, burns resulting in skin wounds[1]. Skin wounds are ubiquitous condition[] where umpteen number of microbes resides which exhibits lagging in recovery and infection. Further, diabetic ulcers healing process cause long-term inflammation[2]. The stages of wound healing includes intricate pathophysiological, cellular and biochemical processes[3]. The most common residers of wound are Staphylococcus aureus, Pseudomonas, Enterococcus and Klebsiella[4] these pathogens will relinquish the healing progress, thus an appropriate environment is essential to promote wound healing [5,6]. It is an well known truth and ample evidence make sure chitosan and green tea plays a remarkable role in the medical history over 200 years.

During the early period of 18th century Buddhist monks recognized he eminent powers of green tea and incorporated in medicinal application[7]. Green tea (Camellia sinensis), an ever-green shrub from Theaceae family[8]. Green tea is a product of dried leaves, one of the most popular beverages consumed worldwide for health promotion since 3000BC [9,10] because of their beneficial polyphenolic compounds which includes Flavins, catechins and polyphenols [11]. Sufficient proof exhibits this plant contains EGCG (Epi-Catchin-3 Gallate) and ECG (Epi- Catchin-3- Gallate) are the most focused antioxidant compounds in greentea, its nearly about 50-60% of the total catechins rate. That attributes for its antioxidant, anticancer and anti-inflammatory properties that helps in resisting collagen production and induce changes in immune response these activities are undertaken by the catechin compound [12,13,14]. That enhances the activity of broad spectrum of antimicrobial activity against both gram positive and gram negative organisms [12]. Those substances balance the collagen production and hence heal the wound [15]. Chitosan, a natural cationic polysaccharide (1-4) 2amino 2deoxy ß-D-glucan[16]. Sources of chitosan includes crustaceans, fungi, algae cell walls, insect exoskeleton & mollusk radulae [17,18]. Among these fish scale is the most underrated, the cheapest and easily available source, about 130 million tones of fish waste is being generated every year around the world [19]. both sea food processing industries and local and harbor fish markets generates a large quantity of waste especially fish scales every year. A major draw back is the lack of waste management, most often fish scales are discarded in ditches, mostly its just left to spoil, leads to environmental pollution [20]. Chitosan is an most promising tool in the field of medical research, it holds prominent biological properties like biocompatibility, non-toxic, antioxidant, anticancer, antimicrobial[21,22]. Generally chitosan extraction process involves three major steps deproteinization, demineralization & deacetylation [23]. Many studies evidence chitosan has the ability of wound healing activity[24]. This study aims at combining the two prime promising substance together . one from plant source and from marine source and assessing their efficacy against the wound pathogens. On the top note an waste material- fish scales have been incorporated in this study which focuses on waste management and applying their functions in medical oriented .

II. MATERIALS

Green tea (Camelia sinensis), marine fish scales, hydrochloric acid ,NaOH, ethanol, Distilled water, soxhelt apparatus, Muller Hinton agar, sterile swabs, nutrient agar, selective media.

1. **Sample Collection – Green Tea:** The *Camelia sinensis* fresh leaves were collected from the tea estate of Munnar, Kerala – Town in India. Washed thoroughly in running water and shade dried completely for 6 days. Motor pulsed cruhed into coarse powder. Stored in an air-tight container.

2. **Fish Scales:** Marine fish scales were collected from Thoothukudi, Tamil Nadu- India, fish market. Thoroyghly washed in running water for several times to remove the dirts attaching to it. Dried under intense sunlight for 5 days.

3. **Wound Sample:** Sterile swabs were used for the collection of wound sample from a 37 years old man (minor injury in leg), collected from Government Hospital, Thoothukui-Tamil Nadu, India. The collected wound samples were placed in sterile nutrient broth and brought to laboratory.

III. METHODOLOGY

1. **Extraction of Chitosan:** For extraction of chitosan, the conventional method was followed. Chitosan ectraction was done following the three major sreps: demineralization, deproteination and deacetylation. For demineralization 50 g of prepared fish scales was treated with 2% hydrochloric acid at solid to solvent ration of 1:5(w/v) for 15 hours with constant stirring at 150 rpm in incubator shaker at room temperature. The residue was washed till the sample reaches neutral pH, the sample was kept for drying at 50^0Cfor overnight. For the second step deproteination, demineralized fish scales was treated with 4% NaOH at solid to solvent ratio 1:5 (w/v) for 24 hours with constant stirring at 150 rpm at 500C in an incubator shaker followed bt complete washing and drying as mentioned above. After this step the resulting end product was chitin. For the final step deacetylation, chitin was treated with strong alkali 50% NaOH for 1g chitin for 1 hour at 70± 5^0C, followes by washing till it reaches neutral pH, after drying at 50±50C for 5 hours [24], the final product recovered was chitosan, the physiological properties were observed and yield calculated. (Table 1)

Table 1: Properties of Chitosan

Physiological parameters	Obsevation
Colour	White
Texture	Powdery
Moisture content	2%
pH	7
Solubility	Acetic acid

2. **Green Tea Extraction:** 30 gram of coarse grinded green tea powder was extracted with 500ml ethanol solvent extraction by using soxhlet extractor in 2 hours [10]. After the extraction, it was filtered and the ethanol solvent was evaporated. DMSO was added, the extracted sample is stored.(Table 2)

Table 2: Sample yield %

Sample	Sample taken (g)	Extracts weight(g)	%yield
Green tea leaves	50	10.16	20.32
Fish scales	50	27.84	55.68

3. **Antibacterial Activity:** The isolated bacterial cultures from the wound samples were spreaded over the Muller Hinton agar plates. chitosan solution (chitosan dissolved in 1% acetic acid) and green tea extract at different concentrations 10µl,50µl,100µl were used in agar well diffusion method. The plates were incubated at 37^0c for 24 hours, zone of inhibition was measured (Table 3)

Table 3: Antibacterial Activity

Zone of inhibition (in mm)						
Organisms	Green tea extract			Chitosan solution		
	10µl	50 µl	100 µl	10 µl	50 µl	100 µl
S.aureus	4 mm	9mm	13.6mm	-	3mm	10.5mm
P.aeruginosa	-	7mm	10.7mm	-	-	8mm
E.coli	3mm	10.4mm	12.3mm	-	10.2mm	11.7mm

4. **Gel formulation:** The following ingredients were measured accurately and formulated in aseptic manner and stored in a sterile container [25] (Table4)

Table 4: Green Tea-chitosan Gel Formulation

Ingredients	Quantity	Role of ingredients
Wool fat	1g	Emollient
Hard paraffin	2.5g	Emollient
Cetostearyl alcohol	2.5g	Emulsifying agent
White soft paraffin	4g	Ointment base
Chitosan	5g	Active ingredient, Cationic biopolymer, antimicrobial,accelerate clotting time,
Green tea extract	5g	Active ingredient, Antimicrobial agent,anti-inflammatory,antioxidant
Total	20g	

5. Evaluation of Ointment

- **Organoleptic test:** The colour, texture, odor of the ointment observed using five senses
- **Measurement of pH:** 10% of the product mixed with 90% of distilled water in a glass beaker, pH paper and pH meter is dipped in it. pH is recorded.
- **Homogenicity Test:** Small amount of the ointment is placed between two glass slides, smeared gently. Homogenecity of an ointment results in the absence of blobs, flat structure, uniform colour of the dot initial till end of the smear.
- **Spreadability:** 0.5 g of the ointment is placed in the middle of round glass plate, the top is covered with another glass plate. To the top of it 100 gram of weight is place for 1 minute. After that the diameter of the ointment spread was measured (Table5).

Table 5: Evaluation of Ointment

Evaluation	Results			
Organoleptic	Colour greenish	Texture Creamy	Odour Odorless	Patch test Non-irritable
pH	6.9			
Homogenicity	Homogenized			
spreadability	3.2cm in diameter			

IV. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION & MINIMUM BACTERICIDAL CONCENTRATION

The MIC and MBC of the formulated green tea-chitosan ointment was assessed using broth dilution method. An inoculum of the bacterial cultures were prepared and suspension was adjusted with a turbidity equivalent to 0.5 McFarland standards. Dilutions of the ointment by two-fold dilution were prepared using sterile Muller Hinton media. One milliliter of cultured suspension was added into each tube. Control tubes contained no ointment. After 24 hours of incubation at 37^0C the test tubes were examined for possible growth and MIC was determined as the lowest concentration that ended with no growth. Tubes without bacterial growth in the MIC test were streaked on nutrient agar plates to achieve MBC tested bacteria. Bacterial growth was observed after incubation, reported as the MBC value [12].

V. RESULTS AND DICUSSION

The bacterial cultures isolated from the wound sample were *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* , they were confirmed with morphological characters and through biochemical tests. The yield of chitosan from marine fish scales was 55.68%, when compared with shrimp and crab shell chitosan it is quite less in yield while comparing with properties and characters it is similar to them [19].At diffecent concentration chitosan showed highest zone of inhibition against E.coli with 11.7mm at 100µl concentration. At lowest concentration 10µl there were no activity. The ethanolic extract of green tea was in dark green colour. One cup of green tea (240ml) may contain 400mg of polyphenolic antioxidants, with up to 200mg of EGCG [13]. The catechin compound exhibits the antibacterial activity, at 100µl concentration showed highest inhibition zone of 13.6mm against *S.aureus*. At 10µl concentration no activity against *P.aeruginosa*. Ointment was

formulated by combining green tea extract and chitosan with several base ingredients and evaluated [25]The results of MIC was 24µg/ml for S.aureus and MBC was 12.5 µg/ml. In conclusion, the current study revealed that integration of the two active ingredients results in inhibiting the bacterial growth.

VI. CONCLUSION

This study conclude that natural substance like green tea and fish scale chitosan are much effective in inhibiting the microbial growth. Thus, it helps in fast wound healing. The formulated ointment has no side effects, used only natural substances one from the plant source and marine waste; it acts as waste conservation factor also.

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