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Research Article

Formulation & Evaluation of Antiseptic Ointment Using Natural Extracts

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Abstract

Wounds are injuries that break the skin. They include cuts, scrapes, scratches, punctured skin or burns. Minor wounds are not usually serious but it is important to clean them. Some wounds heal by themselves without much treatment. However, some require special care or otherwise might catch an infection. Antiseptic creams are meant to prevent wound infection, promote natural wound healing and soothe the skin. However, most of the strong antiseptics available in the market comprise of chemicals that can have harmful effects on the user's skin. Herbal medicines are plant-based drugs that comprise of natural ingredients like leaves, stem, roots, flowers or fruit for medicinal purposes. Such drugs are nontoxic and free from side effects as compared to the synthetic ones. Hence, the project focused on isolation of Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa from wound infections. Extraction of natural extracts from plant parts of Tagetes erecta (marigold), Citrus indica (orange) & Cocos nucifera (coconut) using Soxhlet extraction procedure. The extracts obtained were diluted using Dimethyl sulfoxide and screened for antibacterial activity using Agar cup method. On screening, the antibacterial activity of petals of Tagetes erecta was determined to be the better than of Citrus indica & Cocos nucifera. Hence, an all-natural antiseptic ointment was formulated using Tagetes erecta as the active ingredient. The formulated ointment was evaluated for its physicochemical properties and its antibacterial activity was determined using gradient plate technique.

Key words: *Wound Infection; Natural; Antiseptic; Plant.*

Introduction

involving internal or external break in a living tissue, specifically the dermis of the skin. Minor wounds such as cuts and scratches can be easily treated with proper care. However, in case of wound being infected, it may get worse than better. Wound infection may occur if the pathogenic microbes, especially bacteria grow within the damaged part of the epidermis and cause infections. The skin is the body's initial line of defense and its surface is protected by the acid mantle, which maintains the skin pH and the normal flora, thus, preventing pathogenic microorganisms from invading the skin.**[**3**]** When a person is inflicted by a wound, pathogenic microbes may enter the damaged part of the epidermis and cause wound infection.

Several infections require topical antibiotics to be applied and some might require incision and drainage in order to remove pus and other fluids. Antiseptic creams or first aid creams are applied on the skin or living tissue in order to reduce the possibility of acquiring infection or sepsis.**[**4**]** However, some antiseptics may comprise of alcohols, ammonium compounds, chlorhexidine or some other antibacterial dyes, chlorine and hypochlorite, inorganic iodine compounds, metals, peroxides, permanganates, halogenated phenol derivatives and quinolone derivatives which may cause irritation, chemical burns, rashes and other skin problems if used undiluted or applied to sensitive skin.**[**9**]** Contact with the chemical can cause skin sensitization and allergic contact dermatitis.

Plant drugs are frequently considered to be less toxic and free from side effects that the synthetic drugs cause. Hence, the study focuses on Formulation and Evaluation of an Antiseptic Ointment using Natural Extracts from plants showing antibacterial activity against common bacteria causing wound infections. Specific objectives of the study include Isolation and Identification of Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa from wound infections, exploring the potential of the extracts from Tagetes erecta (marigold) flowers, Citrus indica (orange) peel and Cocos nucifera (coconut) fibers as antibacterial agents against the isolated bacteria, formulation & evaluation of an antiseptic ointment using natural extract showing potential antibacterial activity. The study explains the potential effects of natural extracts against wound infection causing bacteria and would like to promote the shift the focus of the pharmaceutical companies on sustainability and use of natural nontoxic ingredients.

Related Works

S. aureus and P. aeruginosa are the most common bacteria that affect chronic wounds. Serra et al., (2015) carried out a study to isolate these organisms from chronic leg ulcers. Similarly, Bessa et al., (2015) carried out a study to identify the pathogenic bacteria present in wound infections and to study their antimicrobial susceptibility and their resistance profile. The isolated organisms were Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli and Corynebacterium spp. They also reported that polymicrobial infection mainly consisted of two pathogens S. aureus and P. aeruginosa.

Natural extracts of plants have been reported to have antiseptic properties. Almawlah et al., (2017) investigated the antibacterial activity of mint leaves, pomegranate peel and onion bulbs against Pseudomonas aeruginosa isolate from wound infections. Pomegranate peels showed the best antibacterial activity followed by onion bulbs and the least by mint leaves. The antibacterial activity of Ziziphus spinacrist leaves extract against Staphylococcus aureus and Escherichia coli was evaluated by Ali et al., (2013). An effective, stable herbal antibacterial cream was formulated and its physical & antibacterial properties were evaluated. Aruljothi et al., (2014) investigated the antibacterial Activity of Carica Papaya Leaf Extracts against Wound Infection-Causing Bacteria. The leaf extracts were obtained by using acetone, methanol, and water. Baümler et al., (2016) carried out a study to extract the oils from sunflower collets. The solvent used was ethanol and oil was extracted using Soxhlet apparatus. Dasgupta et al., (2012) tested the antimicrobial activity of leaves of marigold (Tagetes erecta) against various Gram negative and Gram-positive bacteria The results suggested that species of Mexican marigold i.e. Tagetes erecta has antibacterial effect against skin infection causing bacteria, and hence can be useful in developing drugs for diseases like dermatitis, acne, skin races and also can be developed as antiseptic. Lobo et al., (2011) in his work described that turmeric and tea tree oil has been well known for their antimicrobial properties. They formulated a cream which had antimicrobial resistance for all the organisms used in the study.

The creams or ointments which are formulated using natural extracts as an active ingredient show high antimicrobial and antiseptic qualities. Yadav et al., (2013) work was aimed to develop an antiseptic cream formulation of Indian basil oil utilizing hydrophilic-lipophilic balance approach. Formulated emulsions were assessed for creaming, droplet size, and turbidity. Cream formulations showed net zone of growth inhibition in the range of 5.0-11.3 mm against bacteria and 4.3-7.6 mm against fungi.

Materials & Methods

Isolation of Bacteria:

Sample collection was conducted by medical officers in an out-patient clinic and in the wards using sterile cotton swabs, following departmental guidelines. Suspensions were prepared by subjecting the cotton swabs with samples into 1ml of sterile saline. A loopful of suspensions was streaked on Sterile Salt Mannitol Agar, Sterile Cetrimide Agar & Sterile MacConkey Agar media plates for isolation of S. aureus, P. aeruginosa & E. coli respectively and the plates were incubated at 37°C for 24 hrs. for E. coli and Pseudomonas and for 48 hrs. for S. aureus. After successful growth of microorganisms, different isolates were re-streaked on specific media to obtain pure cultures. Cultures were sub-cultured and preserved on St. Nutrient Agar Slants.

Identification of Bacteria:

The selected strains were identified based on the cultural, morphological and biochemical characteristics, as outlined in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974). Biochemical identification of the isolates was performed by sugar fermentation test, gelatin hydrolysis tests, nitrate reduction test, starch hydrolysis test, IMVIC tests, and triple sugar iron tests.

Extraction of Natural Extracts Using Soxhlet Extraction Process:

Tagetes erecta flowers, Citrus indica and Cocos nucifera were bought from the local markets. Petals of marigold, peel of oranges and husk fibers of coconut were collected and sun dried for two days. After complete dehydration, the samples were grounded using a simple mixer grinder. 10g of fine powder of each sample was weighed and packed using a filter paper & placed in the thimble. The round bottom flask was filled with solvent and the apparatus was assembled. Extract condensation under reduced pressure and controlled temperature was set to boil through regulated heating mantle. Extraction was carried out for 3-4 hours. The extract was poured into petri plates for evaporation of the solvent and waxy extracts of each sample were obtained after 3-4 days. Dimethyl sulfoxide & Sterile Distilled Water were used as solvent for dilution. 1g each of the extract obtained was dissolved in 10ml of DMSO and the solution obtained was then diluted using sterile distilled water using the following protocol:

Concentration (%)	Extract solution (µl)	Distilled water (µl)
100	200	-
75	150	50
50	100	100
25	50	150

Table 1: Dilution of natural extracts

Evaluation of Antibacterial Activity of Natural Extracts:

A loopful of three bacterial isolates obtained was used and the culture density was adjusted according to McFarland's standard tube no. 1. 0.3ml of culture was added to 20ml Sterile Mueller Hinton Agar butts and the media was poured on to sterile empty petri plates. The media was allowed to solidify and four wells were carved on each plate using a sterilized cork borer. 15µl of extracts at varying concentrations were added to the wells using a micropipette. The plates were incubated at 37° C for 24hrs and zones of inhibition were observed.

Formulation of Antiseptic Ointment:

Category	Material	Composition
Active Ingredient	*Tagetes erecta* (marigold)	1 gm
Cream Base	Beeswax Coconut oil	10 gm 30 gm
Additives	Vitamin E oil Lavender Oil	¼ tsp ¼ tsp

Table 2: Composition of Antiseptic Ointment

Beeswax and coconut oil were melted using a double boiler and allowed to cool. Upon little cooling, the active ingredient was added and stirred. Vitamin E and Lavender oils were added and the resulting mixture was transferred to sterile plastic containers and left to solidify. Thus, an ointment with cream like consistency was prepared.

Physicochemical Evaluation of the Formulated Ointment [13**]:**

- Determination of type of emulsion: Basic fuchsin dye was mixed with cream and one drop of it was taken on a slide and observed under microscope. If the globules appear pink and the continuous phase colorless the ointment is oil in water, in reverse condition, the emulsion is water in oil type.
- **Determination of pH:** The pH of the ointment was determined using a pH paper.
- **Homogeneity:** The formulation was tested for homogeneity by visual appearance and touch.
- **Appearance:** Judged by color, pearlescence and texture observation and graded.
- **Feel after application:** Emollience, slipperiness and amount of residue left after application were checked.
- **Patch Test:** A patch test was performed on sensitive and normal skin types by applying the ointment for various time intervals to observe for burning or itching sensations if any.
- **Removal:** The ease of removal of ointment was tested by washing the applied part with tap water and using cotton pads.
- **Stability Study:** The stability study was carried out by storing the ointment at 8° C and 27º C for various time intervals.

Evaluation of Antibacterial Activity of the Formulated Antiseptic Ointment:

A loopful of three bacterial isolates obtained was used and the culture density was adjusted according to McFarland's standard tube no. 1. Two layers of sterile Nutrient agar were poured successively into a petri plate. The bottom layer consisted of nutrient agar medium with 0.1g of the substance to be tested. The plate was propped up using 2 glass slides, just enough for the agar to cover the entire bottom, forming a wedge that is shallow on one side and deep on the other. Upon solidification, the dish was placed in a horizontal position and another 10 ml of plain sterile Nutrient agar was added, establishing a uniform concentration gradient. The culture suspensions were streaked from the side of low to high concentration and plate was incubated for 24hrs at 37° C and observed for inhibition of growth of bacteria.

Results & Discussions

Isolation of Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa:

Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa were isolated using samples obtained from wound infections. Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa on Sterile Salt Mannitol Agar Medium (SMA), Sterile MacConkey Agar and Sterile Cetrimide Agar Medium respectively. Yellow colored colonies and a surrounding yellow medium were observed indicating growth of coagulase positive S. aureus. Escherichia coli showed pink colored nucleated colonies on the media. Pseudomonas aeruginosa on Sterile Cetrimide Agar medium was observed as white colonies. The morphology of the organisms is described in the table 3.1. The identities of these organisms were confirmed using biochemical tests and sugar fermentation tests detailed in table 3.2. All the results obtained are based on the Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Characteristics	*S. aureus*	*E. coli*	*P. aeruginosa*
Size (mm)	1-2	2	2
Shape	Round	Round	Round
Colour	Yellow	Pink	White
Elevation	Convex	Convex	Convex
Consistency	Butyrous	Butyrous	Butyrous
Opacity	Opaque	Opaque	Opaque
Gram nature	Gram positive	Gram negative	Gram negative
Morphology	Cocci	Rod shaped	Short rods

Table 3.1: Morphology of Isolated Bacteria

Test	*S. aureus*	*E. coli*	*P. aeruginosa*
Catalase	+	+	+
Glucose	+	+	-
Fructose	+	-	-
Lactose	+	+	-
Maltose	+	+	-
Mannitol	+	+	+
Sucrose	+	+	-
Xylose	-	+	-
Indole	-	+	-
Methyl red	+	-	-
Voges-Proskauer	+	-	-
Citrate Utilization	+	+	+
Oxidase	-	-	+
TSI gas	-	+	-

Key: (+) – Indicates Positive Results; (-) – Indicates Negative Results

Table 3.2: Summary of Biochemical Tests

Extraction of natural extracts:

Extraction of natural extracts from petals of Tagetes erecta (marigold), peel of Citrus indica (orange) and fibers of Cocos nucifera (coconut) was done using Soxhlet extraction procedure using ethanol as the solvent. Grounded powders of the above materials were weighed, packed and used for extraction. Upon Soxhlet extraction, natural extracts dissolved in the solvent were obtained. The solutions obtained were kept for evaporation of solvent and pure extracts were collected. Pure extracts of marigold petals and orange peels were observed to be of waxy consistency. Pure extract of coconut fibers was collected in powdered form. The extracts were dissolved in Dimethyl sulfoxide for further antimicrobial studies.

Evaluation of antibacterial activity of natural extracts:

The antibacterial activity of the extracts of Tagetes erecta (marigold), peel of Citrus indica (orange) and fibers of Cocos nucifera (coconut) was done using Agar Cup Diffusion assay. Wherein, the extracts were diluted using sterile distilled water to obtain various concentrations and added to the wells bored into agar medium bulk seeded with the bacteria. The results obtained are presented in the tables 4, 5 & 6. It can be observed that the extract obtained from marigold showed the most inhibition against all three bacteria.

Concentration (%)	Zone of inhibition (mm)		
	Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***
100	17	15	18
75	10	10	12
50	03	04	04
25	00	00	00

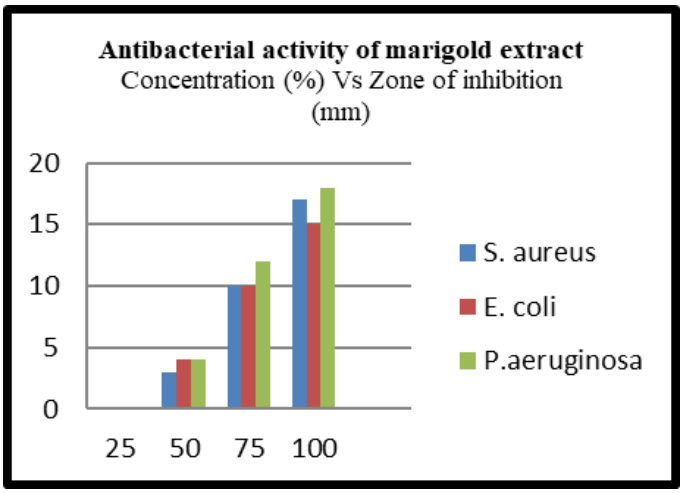
Table 4: Antibacterial activity results for marigold petal extracts

Concentration (%)	Zone of inhibition (mm)		
	Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***
100	04	06	03
75	00	00	00
50	00	00	00
25	00	00	00

Table 5: Antibacterial activity results for coconut coir extracts

Concentration (%)	Zone of inhibition (mm)		
	Staphylococcus aureus	***Escherichia coli***	***Pseudomonas aeruginosa***
100	10	12	09
75	05	08	05
50	00	00	00
25	00	00	00

Table 6: Antibacterial activity results for orange peel extracts



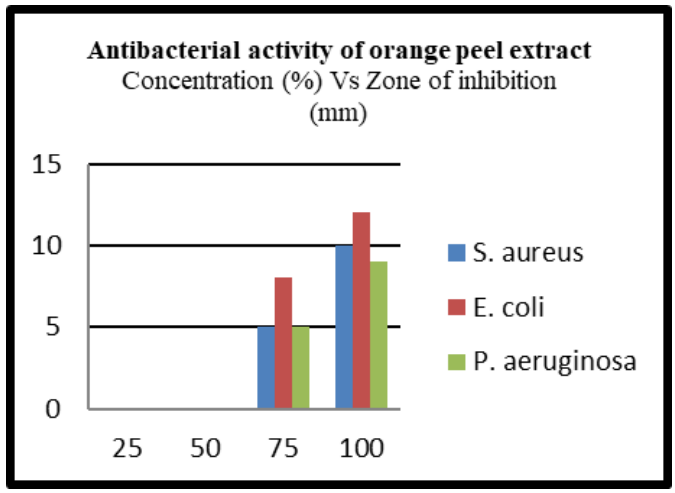


Figure 1: Antibacterial activity of marigold extract **Figure 2:** Antibacterial activity of orange peel extract

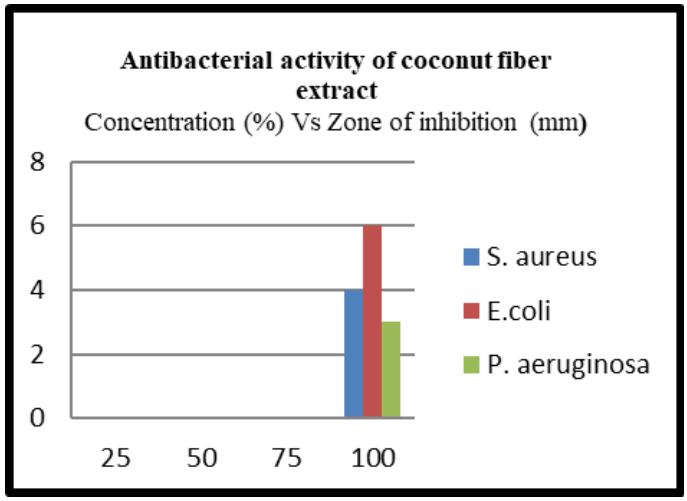


Figure 3: Antibacterial activity of coconut coir extract

Formulation and Physiochemical Evaluation of the formulated antiseptic:

Based on the results obtained, natural extract from petals of Tagetes erecta was chosen as the active ingredient for the antiseptic ointment. The ointment bases were chosen to be coconut oil & beeswax for their skin moisturizing properties. Other additives included Vitamin E oil & distilled Lavender oil for skin nourishment and fragrance respectively. The ointment thus prepared was evaluated based on its physical, chemical and antibacterial properties.

The ointment was naturally yellow in colour and had smooth texture. It was homogenous with water in oil type of emulsion and pH of 5.5. It had good spread-ability and odour with greasy smear. The after feel of the ointment was emollient and slippery. It can be easily removed with a cotton swab or tap water and showed no pigmentation on or after application. Patch tests on sensitive & normal skin types and stability studies at 8°C & 25°C were conducted for various time intervals and the results were noted. The patch test shows no itching or burning sensation on normal and sensitive skin type even after 15 minutes of application. The ointment was stable at 8° and at 25° for up to a month.

Characteristics	Results
Type of Emulsion	Water in Oil
pH	5.5
Homogeneity	Homogenous
Appearance a) Colour b) Pearlescence c) Texture	 Yellow (natural) Lustrous Smooth
Odour	Good
Spread-ability	Good
After feel	Emollient & Slippery
Type of Smear	Greasy
Removal a) Cotton b) Tap water	 Easy removal Easy removal
Pigmentation on Skin a) On application b) After removal	 Absent Absent

Table 7: Physicochemical evaluation of antiseptic ointment

Determination of antibacterial activity of the formulated antiseptic ointment:

Gradient plate technique was used to determine the antibacterial activity of the formulated ointment against previously isolated Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa. Inhibition of growth of Escherichia coli & Staphylococcus aureus was observed in the region of high concentration of the ointment. However, no growth of Pseudomonas aeruginosa was observed in the region of high as well as low concentrations. This indicates that the formulated ointment is capable of inhibiting the growth of Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa isolated from wound infections.

Staphylococcus aureus, Escherichia coli & Pseudomonas aeruginosa were isolated from wound infections using suitable media for growth. Their identity was confirmed on the basis of their morphological, cultural and biochemical characteristics.

Extraction of natural extracts from marigold (Tagetes erecta) petals, orange (Citrus indica) peels & coconut (Cocos nucifera) coir was carried out using Soxhlet extraction procedure using ethanol as solvent. The extracts obtained were poured into petri plates and allowed to dry. Pure extracts obtained after 2-3 days of drying were observed to be waxy consistency. The extracts were diluted using Dimethyl sulfoxide to obtain various concentrations and used for further antimicrobial studies. Antibacterial activity of the extracts against the isolated bacteria was evaluated using agar cup method. Marigold petal extract showed the best antibacterial activity against the isolated bacteria among the three. Hence, marigold was chosen for formulation of the antiseptic ointment.

Marigold extract was used as an active ingredient in the formulation of the antiseptic ointment. Beeswax and coconut oil were used as oil base. Beeswax is a natural ingredient. It does not cause damage to the skin and is used as emollient in creams as it has moisturizing properties. Coconut oil is easy to find and works well with Beeswax. It helps the hard chunks of beeswax to melt easily. Lavender essential oil and Vitamin E were added to improve the fragrance of the ointment.

Physicochemical evaluation of the formulated ointment was conducted. The formulated ointment was yellow in color, smooth in texture, pH 5.5, Water in oil type of emulsion and remained stable at cold as well as room temperature. Patch tests of the ointment showed no irritation on the skin. The antibacterial activity of the ointment was determined using gradient plate technique which showed no growth of Staphylococcus aureus & Escherichia coli in the region of high concentration of antiseptic. Pseudomonas aeruginosa did not show any growth also in the low concentration.

Conclusion

The current study reveals that besides chemicals, plant parts can also be used for formulation of antiseptics and other drugs. It highlights the potential of Tagetes erecta as an antibacterial agent against bacteria causing wound infections. The extract can be used in diverse fields such as developing other antibiotics like the ointment. It can be further separated to determine the active component. Further research can also be carried out on other similar bacteria. The formulated ointment can be tested further to prove its potential as a marketable antiseptic ointment.

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