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Preparation and Evaluation of Antimicrobial and Antioxidant Activity of Polyherbal Ointment

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A B S T R A C T

The main objective of the present study is to formulate and evaluate a poly herbal ointment with antiseptic activity. Ointments were formulated using methanolic extracts of *Eclipta alba, Ocimum sanctum, Azadiracta indica and Achyranthes aspera* which were evaluated for its physicochemical property, antibacterial and antioxidant activity. Ointments were prepared using different concentrations of the extracts such as 2%, 4%, 6% w/w by fusion method using emulsifying ointment as base. Formulations were then tested for its physicochemical properties which gave satisfactory results. The prepared formulations were also stable at 4ºC, 25ºC and 37ºC. Further, Polyherbal formulations were evaluated for its anti-bacterial activity against Betadine (5%w/w) as the standard. All the formulations showed Predominant activity against selected species. Formulations were also evaluated for anti-oxidant activity through reducing power assay, nitric oxide and hydrogen peroxide scavenging method. The results showed that the scavenging activity of the formulations increased with increase in concentration and this is due to the presence of flavanoids and tannins. The presence of both antibacterial and antioxidant activity reveals that the prepared ointment can also be used for wound healing. Hence an attempt was made to formulate a Polyherbal ointment, and to evaluate for its physical parameter, in-vitro anti-oxidant activity and to compare its antibacterial activity with a marketed formulation (5% w/w Betadine).Overall result of this study reveals that this is an effective Polyherbal antiseptic ointment.

Keywords: *Eclipta alba, Ocimum sanctum, Azadiracta indica, Achyranthes aspera* Formulations, Spread ability, Extrudability

A R T I C L E I N F O

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1. Introduction

Preparation of medium and nutrient broth:
Weighed about 0.4g of nutrient broth and dissolved in 30ml of water. Then the broth was suspended in each of test tube. The Muller Hintonagar medium was prepared which contain 9.7g of MHA was suspended in 250ml of water. Then both the medium and broth were for sterilization. After sterilization, the nutrient broth was allowed to cool and then the organism were inoculated and incubated for 4hours.The MHA medium were poured in the Petri dish before cooling and allowed to solidify for about 3-4 hours.

Ayurvedic medicine is a time-tested system of medicine which has been in clinical use for centuries in India. Being a time-tested system, it has an edge over other existing systems of health management [1]. When two or more herbs are used in formulations, they are known as Polyherbal formulations. Ayurveda and herbal medicine has roots in medicinal herbs and they have been practiced for centuries. Herbal medicine is making dramatic comeback and increasing number of patients are visiting alternative medicine clinics. Side effects of synthetic medicine are alarming and recent time has seen risk of herbal and herbal-synthetic drug interactions [2].

In India, from ancient times, different parts of medicinal plants have been used to cure specific ailments. Today, there is widespread interest in drugs derived from plants. This interest primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects. Natural antimicrobials can be derived from plants, animal tissues, or microorganisms. The shortcomings of the drugs available today, propel the discovery of new Pharmacotherapeutic agents in medicinal plants. To determine the potential and promote the use of herbal medicine, it is essential to intensify the study of medicinal plants that find place in folklore [3].

The herbal drugs are boon to our society. These herbal drugs are considered as a therapeutic weapon to fight against various diseases in birds humans and animals, without having any side effects Under the prevailing circumstances further investigations into the concept of Polyherbal formulations should be undertaken .so in the present work, we formulated a Polyherbal ointment with better antimicrobial as well as anti-oxidant activity , can be used for skin infections .In recent years, there has been a great demand for plant derived products in developed countries. The literatures have reported that the usage of the traditional medicines brought a great benefit in skin related diseases. Hence the plant entities derived from the natural source need to be identified and formulated in to suitable dosage form for the management and treatment of various antimicrobial diseases. *Azadirachta indica* known as Neem is well known for its medicinal properties [4]. Its leaves possess broad spectrum of activity against Gram+ve and Gram–ve bacteria including *M.tuberculosis, Vibrio cholera4*.*Ocimum snactum* is known as scared holybsil possess anti-inflammatory, used in treating leprosy, leucoderma, etc [5].

Eclipta alba known as bringeraj antifungal as well as anti microbial activities antioxident6. *Achyranthes aspera* as known as uttareni, and the methanolic extract of the plant have properties like anti bacterial, antifungal activity, anti oxidant activity and anti-inflammatory [6]. The astringent properties of the leaf extracts of *Achyranthes aspera* on the blood vessel has made it a popular plant in the prevention of blood loss from wounds, also its anti-microbial properties has made it a popular choice in disinfecting and treating open wounds [7.] Hence, an effort has been made to establish the scientific validity to investigate the possible antimicrobial and antioxidant activity of the formulated ointments made from the methanolic extracts of the above four herbs. From this investigaton and the results, this polyherbal ointment posses significant antimicrobial as well as anti-oxidant activity can be used for the treatement of burns, wounds, rashes etc.

2. Materials and Methods

Collection of plants: The Leaves of *Eclipta alba, ocimum sanctum, azadiracta indica, achyranthes aspera* species was collected in the month of November at kotappakonda which is near to Narasaraopet, Guntur (Dt), A.P and the collected plants were authentified by Prof. Jayaraman plant anatomist at plant anatomy and research center, thambram, Chennai.

Chemicals and reagents:
Emulsifying wax (I. P, Lobe Chem.), Liquid paraffin (I.P, Nice Chemicals), White soft paraffin (I.P, Lobe

Chem.), Methanol, n-hexane, Sulphuric Acid, Drangendroff's reagent, Molisch's reagent, Acetone.

Equipments:

Soxhlet apparatus, Incubator, Digital balance, Bunsen burner, pH meter, Glass wares, UV Spectrophotometer.

Mediam: Nutrient Agar and Muller Hinton Agar Media

Organisms: *Mucus fungus, Lactobacillus and Escherichia coli.*

Extraction: The collected plants (*Eclipta alba, Ocimum sanctum, Azadiracta indica, Achyranthes aspera species*) were extracted by continuous hot percolation (Soxhletation). 50g of powdered leaves of the above four plants were defatted using petroleum ether. The marc obtained from each of the powdered plant parts were successfully extracted separately with 250 ml of methanol by using Soxhlet apparatus. The extraction was carried out for 24 hours. After extraction, the solvents were distilled out; the concentrated residues were analyzed by chemical tests [8].

Phytochemical analysis:

The methanolic extract obtained after soxhletation was subjected to various phytochemical screening as per the standard procedure to reveals the presence of various active phytoconstituents.[9]

Formulation of ointment:

Working formula (emulsifying ointment base)

Emulsifying wax -300g
White soft paraffin-500g
Liquid paraffin-200g

Procedure:

Required quantities of emulsifying wax, liquid paraffin and white soft paraffin were weighed and melted. To this, adequate quantities of methanolic extract of the mentioned four plants were added and stirred well until a homogeneous mass were obtained [10]. The compositions of different Polyherbal ointment are listed in Table II.

Evaluation:

Physicochemical parameters [11, 12]

Preliminary evaluation of formulations at different concentrations was carried out as follows Colour and odour - examined by visual examination. Loss on drying - determined by placing ointment in Petridish on water bath and dried for 105_0C. The pH of various formulations was determined by using Digital pH meter. One gram of ointment was dissolved in 100 ml of distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values were depicted in Table-III.

Spreadability:

Spreadability of the formulation was determined by an apparatus suggested by Muttimer et al., which was suitably modified in the laboratory and used for the study. It consists of a wooden block, which was provided by a pulley at one end. A rectangular ground glass plate was fixed on this block. An excess of ointment (about 3 g.) under study was placed on this ground plate. The ointment was then sandwiched between this plate and another glass plate having the dimension of fixed ground plate and provided with the hook. A 1 Kg. weight was placed on the top of the two plates for 5 minutes to expel air and to provide a uniform film of the ointment between the plates. Excess of the ointment was scrapped off from the edges. The top plate was then subjected to pull of 80 g. with the help of string attached to the hook and the time (in seconds) required by the top plate to cover a distance of 10 cm. be noted. A shorter interval indicates better Spreadability.

Spreadability is measured as S = M × L /T

M= weight tide to upper slide
L= length of glass slides
T= Time

Extrudability:

A simple method was adopted for this study. The formulations were filled in the collapsible tubes after the ointments were set in the container. The extrudability of the different ointment formulations was determined in terms of weight in grams required to extrude a 0.5 cm of ribbon of ointment in 10 second.

Diffusion study:

The diffusion study was carried out by preparing agar nutrient medium of any Concentration. It was poured into petridish. A hole bored at the centre and ointment was placed in it. The time taken for the ointment to get diffused was noted.

Stability studies:

The stability studies were carried out for the prepared formulations at different temperature conditions (4o C, 25o C and 37o C) for 3 months.

Evaluation of antimicrobial activity [12]

Procedure:

Microorganisms: *Mucus fungus, Lactobacillus, Escherichia coli (aerobic organism).*

Standard used: 5% w/w Betadine ointment.

Sample preparation: About 10mg of ointments (2%, 4%, 6% w/w) were weighed and dissolved in DMF (dimethyl formamide) and used for activity studies.

Preparation of medium and nutrient broth:

Weighed about 0.4g of nutrient broth and dissolved in 30ml of water. Then the broth was suspended in each of test tube. The Muller Hintonagar medium was prepared which contain 9.7g of MHA was suspended in 250ml of water. Then both the medium and broth were for sterilization. After sterilization, the nutrient broth was allowed to cool and then the organism were inoculated and incubated for 4hours.The MHA medium were poured in the Petri dish before cooling and allowed to solidify for about 3-4 hours.

Methodology:

The bacterial culture was spread on the culture medium and a well was bored in the middle of the agar. Then different samples and standard solutions of 0.05ml was poured inside these wells and plates were incubated at 37oC overnight for observation. The presence of inhibition was noted and compared with the control. The susceptibility of the test organism to the tested plant extract was determined by observing the zone of inhibition around each well

***In-vitro* antioxidant study: [13, 14]**

Reducing power assay method

Reagents:

0.1%Ferric chloride, 1%Ferricyanide, Phosphate buffer6.6, Trichloro acetic acid.

Preparation of standard ascorbic acid solution:

Weighed accurately 50mg of ascorbic acid and made up to 50ml with methanol.

Preparation of sample solution:

50mg of ointments (2%, 4%, 6% w/w) was dissolved in 50ml of methanol. From the above, different concentrations (0.5, 1, 2, 3, 4, 5ml) were pipetted out and made up to 10ml with methanol. Added 2.5ml of phosphate buffer 6.6 and 2.5ml of potassium ferricyanide to each of the test tubes and was incubated at 40oC for 20min. After incubation, 2.5ml of Tri chloro acetic acid was added and centrifuged the reaction mixture for 5min.To 2.5ml of this reaction mixture 0.5ml ferric chloride and 2.5ml water were added. The absorbance was measured using Double beam spectrophotometer at 700nm.

Preparation of control:

10ml of methanol was taken. To it 2.5ml of potassium ferricyanide and 2.5ml of buffer 6.6 were added. The above reaction mixture was incubated at 40oC for 20min and centrifuged for 5min.To this add 0.5ml ferric chloride and 2.5ml of water.

Nitric oxide scavenging method

Reagents:

Sodium nitroprusside, phosphate buffer solution, Griess reagent were procured. Fine chemicals and all the solvents used were of A R grade.

Preparation of test sample:

50 mg of ointments was dissolved in 50ml of distilled methanol to obtain a solution of 1mg/ml. From this stock solution, different working dilution were prepared to get concentration of 100, 200, 300, 400, 500 µg/ml.

Methodology:

Nitric Oxide was generated from sodium nitroprusside was measured by the Griess reagent. Sodium nitroprusside5mM in phosphate buffer of pH 7.4 saline was mixed with different concentrations of the ointments (100, 200,300,400,500 µg/ml) dissolved in water and incubated at 25 ºC for 150 minutes. At different time interval, sample (1.5 ml) of the incubated solution were removed and diluted with 1.5 ml Griess reagent (1%sulphanilamide, 2% H3PO4, and 0.1%naphthylethylenediamene dihydrochloride).

Evaluation: The absorbance was read at 564nm. Ascorbic acid was used as the standard. The difference in the absorbance between test and control of Nitric Oxide were calculated and expressed as percent scavenging of Nitric Oxide radical. Capability to scavenge the nitric oxide radical was calculated by using equation.

Hydrogen peroxide scavenging activity:

Solution of hydrogen peroxide (40mM) was prepared in phosphate buffer of the pH 7.4.The concentration of hydrogen peroxide was determined by absorption at 230nm using spectrophotometer. The ointments 1mg/ml in methanol were added to hydrogen peroxide solution (0.6ml, 40mM).The absorbance at 230nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The % of the hydrogen peroxide scavenging by the samples and the standard compounds were calculated.

3. Results and Discussion

Literatures revealed that the selected four herbs *Azadirachta indica, Mimosa pudica, Chromolaena odorata, Samadera indica* have antioxidant and antibacterial activity. Hence an attempt was made to formulate a polyherbal ointment, and to evaluate for its physical parameter, in vitro antioxidant activity and to compare its antibacterial activity with a marketed formulation (5% w/w Betadine).Extraction and the phytochemical screening was done using methanol as the solvent. Phytochemical screening confirmed the presence of various phytoconstituens like carbohydrate, glycosides, flavanoids and tannins. In the present study, Polyherbal ointments were prepared by fusion method using emulsifying ointment as the base. The formulations were then evaluated for their physical parameters, *In-vitro* antioxidant and compared with marketed 5%w/w Betadine ointment for its antibacterial activity. These physical parameters were within the acceptable range.

The stability studies were carried out and inferred that the formulations showed no signs of instability. The antibacterial activity of prepared ointments were compared with 5%w/w Betadine ointment using selected species of microorganism such as *Mucus fungus, Lactobacillus, Escherichia coli (aerobic organism)* and it showed that formulations like F2 and F3 showed greater activity Staphylococcus aureus and Bacillus sp compared to 5% Betadine. So, antimicrobial study shows that the prepared ointments has better activity *Mucus fungus, Lactobacillus, Escherichia coli (aerobic organism)* compared to standard 5% Betadine ointment. Anti-oxidant activity inferred that the formulated ointments showed similar activity as that of standard ascorbic acid and hence revealed that this activity is due to the presence of flavanoids and tannins. Hence the study concludes that an efficient antiseptic ointment with antimicrobial and antioxidant activities can be formulated from the methanolic plant extracts of *Eclipta alba, ocimum sanctum, azadiracta indica, achyranthes aspera* which can also be used forwound healing and various skin infections.

Table 1: Composition of Polyherbal Ointments

Ingredients	F1 (2%)	F2 (4%)	F3 (6%)
Azadirachta indicam ethanolic extract	2g	4g	6g
Ocimum sanctummethanolic extract	2g	4g	6g
Eclipta alba methanolicextract	2g	4g	6g
Achyranthes aspera methanolic extract	2g	4g	6g
Emulsifying ointment	q.s to 100g	q.s to 100g	q.s to 100g

Table 2: Phytochemical Screening of the Methanolic Extract of *Poly Herbal Extract*

Chemical Constituents	**Polyherbal extract**
Carbohydrates	+
Proteins	-
Alkaloids	+++
Saponins	-
Tannins	++
Flavonoids	++
Steroids	+
Triterpenoids	+
Glycoside	+++

Table 3: Physicochemical evaluation of formulated formulations

Physicochemical parameters	**F1 (2%)**	**F2 (4%)**	**F3 (6%)**
Colour	Dark green	Dark green	Characteristic
Odour	Characteristic	Characteristic	41%w/w
Loss of drying	37%w/w	40%w/w	6.6
PH	7.02	6.88	13
Spread ability (Seconds)	12	12	182g
Extrudability	180g	180g	0.7cm
Diffusion study (after 60 min)	0.7cm	0.8 cm	Stable
Storage(4ºC, 24ºC, 37ºC)	Stable	Stable	Characteristic

+present, - absent

Table 4: Antimicrobial activity of formulated ointment

Ointment	***E. coli***	***Mucus fungus***	***Lactobacillus***
Herbal Ointment	1.25± 0.2	1.50±0.12	1.11±0.32
Polyherbal Ointment	1.85±0.42	1.93±0.31	1.71±0.13
Standard (Gentamycin)	2.21±0.23	2.00±0.11	2.11±0.23
Control	0.166±0.11	0.41±0.23	0.08±22

Diameter of Zone of inhibition in cm

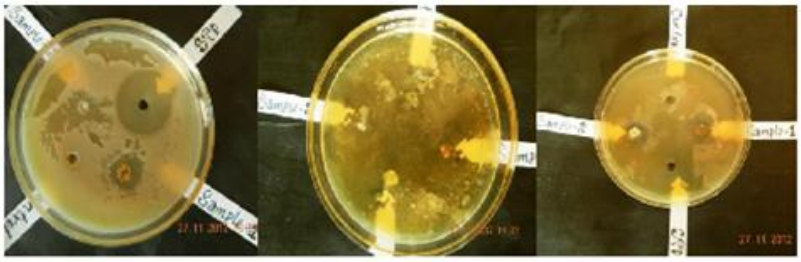

Figure 1: Antimicrobial activity of formulated ointment

Table 5: Antioxidant activity of formulated ointments by reducing power assay

Concentration (µg/ml)	**Absorbance**			
	Standard	**F1 (2%)**	**F2 (4%)**	**F3 (6%)**
50	0.346	0.213	0.156	0.222
100	0.369	0.26	0.21	0.253
200	0.412	0.276	0.313	0.392
300	0.578	0.346	0.51	0.472
400	0.687	0.41	0.682	0.593
500	0.786	0.511	0.703	0.639
Control=0.992				

Table 6: Antioxidant activity of formulated ointments by nitric oxide scavenging activity

Concentration (µg/ml)	Absorbance				% Inhibition			
	F1	F2	F3	STD	F1	F2	F3	STD
100	0.637	0.641	0.637	0.613	60.67	60.43	60.67	62.16
200	0.624	0.628	0.626	0.603	61.48	61.23	61.35	62.39
300	0.619	0.624	0.613	0.586	61.79	61.48	62.16	63.83
400	0.598	0.615	0.605	0.562	63.08	62.03	62.65	65.31
500	0.548	0.589	0.569	0.525	66.17	63.64	64.87	67.59

Table 7: Antioxidant activity of formulated ointments by hydrogen peroxide scavenging activity

Concentration (µg/ml)	Absorbance				% Inhibition			
	F1	F2	F3	STD	F1	F2	F3	STD
100	1.179	0.61	0.515	0.495	45	71.5	76	76.95
200	0.77	0.456	0.451	0.394	64.13	78.76	78.99	81.64
300	0.551	0.427	0.441	0.295	74.33	80.11	79.45	86.25
400	0.455	0.369	0.399	0.195	78.8	82.81	81.41	90.91
500	0.372	0.366	0.376	0.134	82.67	82.95	82.48	93.75

Figure 2: Plant Images Which Are Used For Poly Herbal Formulation

4. Conclusion

A combinational therapy is the need of hour to treat eczema and pruritis. This can be achieved by Clotrimazole and Ichthammol (an antifungal and antiseptic).In this study, ointment was formulated with different bases like white soft paraffin, cetostearyl alcohol, hard paraffin, and light liquid paraffin. By combining these drugs with appropriate ointment bases (as polyherbal formulation) a better therapy and patient compliance can be achieved. Both the formulations may shows the batter way to use these drugs with more significant way and more over WHO also emphasizes the herbal medicine for treatment. This study showed the convenient preparation and more effective use of both the herbs with modernized formulations with comparatively old form of medicine. This affords may leads a beginning of proper utilization and conservation of medicinally important herbs and cost effective treatment in future.

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