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Formulation and evaluation of an effective pH balanced topical antimicrobial product containing tea tree oil

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The effect of pH on the antimicrobial activity of *Melaleuca alternifolia* essential oil formulations was studied. Microemulsions, liposomal dispersions, multiple emulsions and a colloidal bed of sterile clay were formulated using 5% w/w of tea tree oil. A number of formulations were prepared at various pH values (5.0, 5.5, 6.0, 6.5, and 7.0). Thermal stability studies showed that the formulations were stable for more than eight months. Agar dilution tests showed MICs of 1.0% v/v *S. aureus* and *S. epidermidis*. In the broth dilution test, MBC of the oil for *P. acnes* was 0.5% v/v. MIC and MBC values were comparable to those of non-formulated tea tree oil, indicating that tea tree oil retained its activity in the above-mentioned formulations. The microbiological evaluation showed that the formulations containing 5% w/w tea tree oil had a maximum effect at pH 5.5.

1. Introduction

Tea tree oil is obtained from various members of the *Melaleuca* genus (tea-tree). The most common species used is *Melaleuca alternifolia*, and the oil is obtained by steam distillation of the leaves (Altman 1988). Tea tree oil is a popular component of skin preparations, and a number of its suggested uses imply an antimicrobial effect (Hammer et al. 1998, Harkenthal et al. 1999). Studies demonstrating the antimicrobial activity of the oil have been reviewed and tea tree oil is recommended for the treatment of acne vulgaris. *Propionibacterium acnes* and coagulase-negative *Staphylococci* have been implicated in the aetiology of acne vulgaris and it is expected that the oil works by eradicating these microorganisms from acne lesions. A study comparing tea tree oil gel to benzoyl peroxide lotion demonstrated the efficacy of the oil for treating the dermal condition (Bassett et al. 1990).

The pH of the skin follows a sharp gradient across the stratum corneum (SC), which is expected to play an important role in controlling the enzymatic activities involved in cellular metabolism and renewal. This gradient is maintained by several systems, such as sweat and sebum secretion and degradation as well as cellular metabolism. The pH of some dermal products might influence the antimicrobial activity and also have detrimental effects on enzymatic processes in the corneal layers. Such a process affects the natural defense mechanism of the skin, which plays a critical role in the efficacy of the product. The objective of the present study was to examine the *in vitro* antimicrobial activity of tea tree oil and tea tree oil formulations against *S.aureus, S. epidermidis* and *P. acnes*, and to study the role of pH in the antimicrobial activity against these microorganisms.

2. Investigations and results

The MICs of the oil for *S. aureus* and *S. epidermidis* were 1.0%. The MBC of the oil for *P. acnes* was 0.5%. Tea tree oil formulations showed MICs and MBCs similar to the non-formulated tea tree oil. Thermal stability studies showed that the formulations containing tea tree oil were stable for more than eight months. The microbiological studies showed that the formulations containing 5% tea tree oil exhibited the maximum zone of inhibition at pH 5.5. The diameter of zone of inhibition (mm) at pH 5.5 against *S. aureus* (NCIM No. 2079), *S. aureus* isolate, *S. epidermidis* (NCIM No. 2493), *S. epidermidis* isolate and *P. acnes* (MTCCNo. ∗1951) in different formulations are shown in Tables 1–4. Comparing various tea tree oil formulations as mentioned above, it was seen that microemulsion exhibited the highest antimicrobial activity, followed by liposomal dispersion, multiple emulsion and then colloidal bed formulations. The enhanced antimicrobial activity of the microemulsion may be due to the smaller particle size of these formulations, which may have resulted in better diffusion through all barriers. A slight increase in pH from 5.0 to 5.5 showed an increase in antimicrobial activity for all the formulations. But in contrast the antimicrobial activity decreased when the pH was increased over the slightly acidic pH of 5.5 to neutral pH.

3. Discussion

The antimicrobial activity comparing the MICs and MBCs of tea tree oil and tea tree oil formulations against various organisms demonstrated that tea tree oil in formulation appears to retain its activity. *P. acnes* was more sensitive to the oil compared to *Staphylococci*, which is in agree-

Table 1: Effect of pH on the antimicrobial activity of microemulsion

pH levels	Diameter of zone of inhibition (mm) ± S.D				
	S. aureus (NCIM No. 2079)	*S. aureus* isolate	*S. epidermidis* (NCIM No. 2493)	*S. epidermidis* isolate	*P. acnes* (MTCCNo. ∗1951)
pH 5	14.8 ± 0.16	14.4 ± 0.17	14.9 ± 0.55	14.2 ± 0.11	16.4 ± 0.56
pH 5.5	15.6 ± 0.82	14.9 ± 0.35	15.4 ± 0.27	14.6 ± 0.25	17.4 ± .51
pH 6	14.6 ± 0.26	14.3 ± 0.49	14.4 ± 0.56	14.2 ± 0.14	16.2 ± 0.29
pH 6.5	13.9 ± 0.73	13.5 ± 0.44	13.9 ± 0.41	13.7 ± 0.25	15.9 ± 0.47
pH 7	13.1 ± 0.54	13.0 ± 0.18	13.2 ± 0.30	13.2 ± 0.41	15.9 ± 0.17

Table 2: Effect of pH on the antimicrobial activity of tea tree oil liposomal dispersion

pH levels	Diameter of zone of inhibition (mm) ± S.D				
	S. aureus (NCIM No. 2079)	*S. aureus* isolate	*S. epidermidis* (NCIM No. 2493)	*S. epidermidis* isolate	*P. acnes* (MTCC No. ∗1951)
pH 5	14.0 ± 0.36	13.9 ± 0.17	14.2 ± 0.15	14.0 ± 0.43	15.4 ± 0.56
pH 5.5	14.6 ± 0.64	14.6 ± 0.35	14.8 ± 0.63	14.5 ± 0.26	16.6 ± 0.51
pH 6	13.8 ± 0.57	14.0 ± 0.49	13.8 ± 0.56	14.0 ± 0.17	15.7 ± 0.29
pH 6.5	12.9 ± 0.44	12.8 ± 0.14	13.2 ± 0.41	12.9 ± 0.25	15.7 ± 0.17
pH 7	12.6 ± 0.40	12.5 ± 0.48	13.0 ± 0.30	12.9 ± 0.11	15.0 ± 0.47

ment with earlier reports (Carson and Riley 1994; Raman et al. 1995). Thermal stability studies showed that on exposure to different climatic conditions, the temperature did not significantly affect the formulations. The microbiological studies supported the classical attribution of merits to the skin's acid mantle, which is in coincidence with the concept that most bacteria grow better around neutral pH. Acidic, or slightly acidic pH could be bacteriostatic for some strains (Lukacs 1992) and many deodorants are developed to control the pH to a value around 5.5 (Rennie et al. 1990). The organic acids contained in normal sweat have been reported to exert a fungistatic or bacteriostatic activity, which is dependent on the pH. Similarly, 'the water soluble constituents of the horney layer' kill some *Staphylococcus* species at pH values around 5.5, but not at pH 7.0 (Parra and Paye 2003). This slightly acidic pH on the skin surface is comparable with the optimum pH of the formulation, which acts against invading microorganisms. The slightly acidic pH of the formulation in addition to the acid mantle of the skin probably influences the ionization of compounds in the SC, mainly weak acids and bases. Such a process is assumed to be important in the modulation of the enzymatic activity involved in the extracellular processing of lipids and proteins between the stratum granulosum and stratum compactum. The formulations at pH 5.5 have a two-way mechanism. In addition to the maximum antimicrobial activity they may play a critical role in the creation of a "good" skin surface, thereby increasing the natural defense mechanism of the skin. Demonstrating that products were active *in vitro* suggests that these formulations are potentially useful *in vivo*. However *in vivo* effectiveness can only be determined with a randomized clinical trial to evaluate each product thoroughly. It is also possible that product ingredients other than tea tree oil may have antibacterial activity and are contributing to the total activity of the product. However this is unlikely as the tea tree oil MIC's were the same for pure tea tree oil and for the products investigated.

Table 3: Effect of pH on the antimicrobial activity of tea tree oil multiple emulsion

pH levels	Diameter of zone of inhibition (mm) ± S.D				
	S. aureus (NCIM No. 2079)	*S. aureus* isolate	*S. epidermidis* (NCIM No. 2493)	*S. epidermidis* isolate	*P. acnes* (MTCC No. ∗1951)
pH 5	13.6 ± 0.28	13.5 ± 0.26	13.3 ± 0.11	12.9 ± 0.39	14.0 ± 0.47
pH 5.5	14.1 ± 0.15	13.7 ± 0.19	14.2 ± 0.60	13.9 ± 0.67	15.0 ± 0.16
pH 6	13.0 ± 0.21	12.7 ± 0.36	13.0 ± 0.46	12.7 ± 0.54	14.2 ± 0.33
pH 6.5	12.2 ± 0.39	12.0 ± 0.24	12.2 ± 0.23	12.0 ± 0.76	13.6 ± 0.22
pH 7	12.2 ± 0.27	11.9 ± 0.06	12.0 ± 0.18	11.8 ± 0.79	13.2 ± 0.14

Table 4: Effect of pH on the antimicrobial activity of tea tree oil colloidal bed

pH levels	Diameter of zone of inhibition (mm) ± S.D				
	S. aureus (NCIM No. 2079)	*S. aureus* isolate	*S. epidermidis* (NCIM No. 2493)	*S. epidermidis* isolate	*P. acnes* (MTCC No. ∗1951)
pH 5	12.8 ± 0.45	12.4 ± 0.63	12.6 ± 0.13	12.4 ± 0.25	13.0 ± 0.38
pH 5.5	13.0 ± 0.56	13.0 ± 0.23	13.1 ± 0.19	13.0 ± 0.19	13.6 ± 0.14
pH 6	12.2 ± 0.14	11.9 ± 0.30	12.6 ± 0.23	12.0 ± 0.51	12.9 ± 0.25
pH 6.5	11.6 ± 0.58	11.6 ± 0.52	11.8 ± 0.58	11.8 ± 0.34	12.6 ± 0.39
pH 7	11.4 ± 0.27	11.2 ± 0.17	11.4 ± 0.45	11.4 ± 0.17	12.6 ± 0.36

One issue that may require further investigation is that of adverse reactions to tea tree oil. The lipophilic nature of tea tree oil, which enables it to penetrate skin, not only potentiates the antimicrobial action but also the possibility of toxicity due to dermal absorption. When tested in rabbits with prolonged skin contact at a dose of $2\,g \cdot kg^{-1}$ no toxicity was observed. Some irritation resulted when neat oil was applied to intact and abraded skin but no skin irritation occurred when 25% oil was applied to shaved rabbit skin for 30 days (Carson and Riley 1993). A wutact allergen has recently been identified (Harkenthal et al. 2000). Bassett et al. 1990, investigated the use of tea tree oil in the treatment of acne vulgaris and found no skin irritation in trial patients. The incidence of adverse reactions to tea tree oil in the general population is as yet unknown, but these studies suggest that the incidence of skin irritation may be relatively low.

In summary, tea tree oil formulated at the right pH showed significant promise as a potential therapeutic agent for the treatment of acne vulgaris. *In vitro* results indicate susceptibility at low concentration and products containing tea tree oil maintained their *in vitro* efficiency. What is now required are clinical trials to determine the usefulness of tea tree oil *in vivo*.

4. Experimental

4.1. Materials

Tea tree oil was purchased from Blossom Kochhar beauty products, New Delhi, India. Isopropyl myristate, Tween®-80, Span®-80, toluene, ethyl acetate, methanol, acetic acid, sulphuric acid and propylene glycol were purchased from S.d. fine – Chemicals Ltd., Mumbai, India. Soybean lecithin, nutrient agar, glycerol monostearate, citric acid, potassium hydroxide, calamine, kaolin, bentonite, methyl paraben and glycerol were purchased from E. Merk, Mumbai, India. If otherwise indicated, material concentration is expressed as percentage by weight (w/w).

4.2. Test organisms

S. aureus NCIM No. 2079 and *S. epidermidis* NCIM No. 2493 were cultured on nutrient broth and incubated for 24 h at 37 °C (overnight culture, ONC).

P. acnes MTCC No. *1951 was cultured on blood agar or in brain heart infusion (BHIB) and incubated in an anaerobic chamber at 35 °C in an atmosphere consisting of 10% CO_2, 10% H_2 and 80% N_2 for 48–72 h.

4.3. Tea tree oil formulations.

A microemulsion was prepared using 23% isopropyl myristate, 5% tea tree oil, 42% Tween®-80, 6% glycerol and 24% water. The microemulsion was prepared by slow trituration of the aqueous glycerol solution in isopropyl myristate, tea tree oil – Tween®-80 mixture. Trituration was carried out until the mixture became hazy or turbid to establish the region of clear isotropic mixtures (Rosano et al. 1988). Phase studies showed that a glycerol-to-water mass ratio of 2 : 8 at 30% w/w total concentration of glycerol and water and isopropyl myristate-tea tree oil to Tween®-80 mass ratio of 2 : 3 produced the largest microemulsion region and this ratio was selected for the preparation of microemulsion for further experiments. The particle size of the formulation was determined using a Malvern particle size analyzer. The microemulsion had a mean particle size of 30 ± 14.6 nm.

A liposomal dispersion was prepared by dispersing 1.25% phosphatidyl choline and 0.25% glyceryl stearate citrate in 8.5% propylene glycol. The resulting clear solution was then slowly diluted with 10% water to yield a lamellar liquid crystalline phase (Diec et al. 2002). Tea tree oil 0.5% was added to the lamellar phase. Upon addition of water to reach 100% composition, the system was converted into vesicle dispersion. The resulting liposomal dispersion was examined using a Nikon Diaphot light microscope to assure uniformity of the liposomal preparation. The liposomal dispersion was sonicated for 10 min and centrifuged (30000 rpm) and the encapsulation efficiency was determined by analyzing the supernatant by HPTLC. The liposomal formulation was found to have 60% encapsulation efficiency and sufficient liposomal pellets were redispersed in water to prepare a liposomal dispersion containing equivalent of 5% tea tree oil. The particle size of the liposomal dispersion was determined using a Malvern particle size analyzer. The particle size of the liposomes was 320 ± 24.3 nm.

Multiple emulsions were prepared by dispersing 60% of the primary emulsion into the external oil phase of isopropyl myristate containing 5% tea tree oil. The primary emulsion was prepared using 37% isopropyl myristate, 5% tea tree oil, and 6% Tween®-80 with 52% water as the intermediate aqueous phase. The primary emulsion was prepared at high-speed homogenization of 2000 rpm and the secondary emulsion at a low speed of 800 rpm. Simple and multiple emulsions were examined under the light microscope to determine the characteristics of the globules (Filho 1997). Microscopic analysis showed the presence of both primary and multiple characters. A malvern particle size analyzer determined the globule size distribution. Globule sizes of 5.3 ± 0.8 μm for the primary emulsion and an average size of 10 ± 1.2 μm for the multiple emulsion were observed.

Colloidal bed formulations are pastes or creams, sometimes described as mudpacks, applied to the face, prepared using kaolin, calamine and bentonite, which are termed as 'clays' (Hunting 1993). The formulation was prepared using 34% kaolin, 10% calamine, 6% bentonite, 3.5% glyceryl monostearate, 0.25% methyl paraben, 10% glycerine, 5% tea tree oil and water to make 100% composition. The clays were presterilised at 150 °C in a dry heat oven for 1 h. Water and glycerin were added to the clays along with glyceryl monostearate and methyl paraben and mixed well. Tea tree oil was dispersed with Tween®-80 and mixed until a fine paste was obtained. The particle size as determined by a Malvern particle size analyzer was 50 ± 16.2 μm.

A number of formulations were prepared using tea tree oil at various pH levels using citric acid and potassium hydroxide (5.0, 5.5, 6.0, 6.5, and 7.0) for all the formulations.

4.4. Thermal stability tests

Tests for phase separation and visual microbial contamination were carried out on the formulations like microemulsion, liposomal dispersion, multiple emulsion and colloidal bed, which were kept at 4 ± 1 °C, room temperature (30 ± 1 °C) and 40 ± 1 °C.

4.5. Microbial evaluation (S. aureus and S. epidermidis)

Agar dilution technique: Oil sample (1 ml) was added to sterile molten Mueller Hinton (20 ml) in sealed bottles, shaken well and left overnight at 55 °C to allow mixing. Tween®-80 (0.5%) was added to facilitate even dispersion of the oil. Dilutions were prepared to give concentrations of the oil in the range of 0.2–5% v/v. Plates were prepared from each dilution and they were inoculated with approximately 1×10^6 cfu ml^{-1} of ONC of *S. aureus* and *S. epidermidis.* The presence or absence of growth of the organisms was noted after overnight incubation at 37 °C. Each test was performed in triplicate.

4.6. Microbial evaluation (P. acnes)

Broth dilution test: Concentrations of tea tree oil ranging from 0.2–5% v/v were prepared in BHIB. Tween®-80 (0.5%) was added to the broth to facilitate even dispersion of the oil throughout the growth medium. Broths were pre-reduced in the anaerobic chamber for 4–6 h prior to inoculation. Overnight cultures of *P. acnes* strains were used as the inoculum after the cell density was adjusted to approximately 10^8 cfu ml^{-1}. Viable counts were used to confirm the concentration of *P. acnes.* Broths containing tea tree oil were inoculated so that the final concentration of *P. acnes* was approximately 1×10^6 cfu ml^{-1} in each tube. Inoculation was performed in the anaerobic chamber and the tubes were incubated at 35 °C for 36–48 h. After incubation, the number of *P. acnes* in each broth was determined by viable counts on blood agar plates.

4.7. In vitro testing of tea tree oil products

Formulations containing 0.2–5% v/v of tea tree oil were prepared. The formulations were inoculated with *S. aureus, S. epidermidis* and *P. acnes.* After incubation as mentioned earlier for the respective organisms, samples were taken and spread on Mueller Hinton and blood agar and then MICs and MBCs were determined as described above.

4.8. Effect of pH on the antimicrobial activity of tea tree oil formulations

Patients reporting for treatment of facial acne vulgaris provided clinical specimens for the bacterial isolates used in this investigation. Type cultures (*S. aureus* NCIM No. 2079, *S. epidermidis* NCIM No. 2493 and *P. acnes* MTCC No. *1951) were also used. Specimens from infected sites were taken using sterile cotton tipped applicators. These specimens from facial acne were taken after treating the surface of the skin with a cleansing lotion. The specimen samples were immediately applied to nutrient agar and were streaked to obtain isolated colonies. They were gram stained and later taken up for presumptive identification and confirmation of the test organisms.

Aerobic organisms: A 0.2 ml volume of overnight nutrient broth culture of *S. aureus* and *S. epidermidis* was seeded into 20 ml of nutrient agar. Cups were bored using sterile cork borers. One Pasteur pipette drop of molten agar (45 °C) was placed into the cups to seal the bottom. 200 mg of the formulations containing 5% tea tree oil were placed into the cups, allowed to stand for 1 h and incubated at 37 °C. The diameter of zone of inhibition was measured after 24 h incubation.

Anaerobic organisms: One loop full of *P. acne* was streaked on 20 ml of blood agar. Cups were bored using sterile cork borers. One Pasteur pipette drop of molten agar was placed into the cups to seal the bottom. 200 mg of the formulations containing 5% tea tree oil were placed into the cups and incubated in an anaerobic jar. The diameter of zone of inhibition was measured after 48 h.

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