SOLUBILIZING ESSENTIAL OILS AND PREPARING STABLE, TRANSPARENT AND STAIN-FREE LIQUID ROLL-ON PREPARATION FOR FOOT APPLICATION

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ABSTRACT: Sweating is a biological mechanism that helps to synchronize temperature of human body. Foot odor is produced by metabolism of gram-positive bacteria such as *Staphylococcus epidermis, Bacillus subtilis and Propionibacterium Acnes*. Microbial vegetation present over foot-skin causes break-down of protein as well as lipid containing secretion of exocrine glands. Enzymes responsible for break-down of lipids and proteins are proteases and lipases. It disrupts the protein and lipids into free fatty acids (short-chain) and amino-acids. These are eventually vaporized in atmosphere owing to its type of chemical. Further, such compounds which are volatile in nature finally move to receptors of nose and are noticed as unlikable odorants. Few spray products containing natural oil/s are available. However, these products have stability issue due to phase separation, and it produce stain on foot skin or apparel.The present study focussed on solubilizing the essential oil/s and tested susceptibility of *Bacillus subtilis* and *Staphylococcus epidermidis* to oil combinations. The outcome showed that a combination of lemon oil, neem oil and tulsi oil possessed synergistic antibacterial activity. A foot deodorizing liquid roll-on was prepared using different solubilizers and tested. The liquid roll-on preparation showed desired properties such as stability, clarity, consistency, spreadability, quick absorption post application, non-stickiness and non-dryness, and absence of residue. The foot preparation demonstrated antibacterial efficiency against the bacteria responsible for producing a strong foot odour.

KEYWORDS: Foot odour; Lemon oil; Tulsi oil; Neem oil; Deodorant; liquid roll-on *Bacillus subtilis;Staphylococcus epidermidis*.

1. INTRODUCTION

Foot odour is known to be triggered by the secretion of glands such as eccrine and sebaceous glands. The secretion contains various amino acids, including serine, alanine, leucine,

isoleucine and valine[1]. Amongst these amino acids, valine, leucine, and isoleucine are accountable for forming foot odour; serine and alanine are considered basic amino acids responsible for moistening sweat[1,2]. The amino acids such as leucine, valine, and isoleucine are broken down by microorganisms present on the skin surface into lower fatty acids, which are volatile in nature. It is known that gram-positive bacterial metabolism causes a strong foot odour. Microbial enzymes such as proteases or lipases disrupt the secretion of protein and lipids into fatty acids and amino acids that get vaporized. These volatilized compounds are perceived as unpleasant odorants. Amoore and Kanda *et al*., [3,4] found that isovaleric acid appears to be a crucial odorant. Further, Sawano[5]found that foot odour consists of isovaleric acid and various free fatty acids such as propionic, isobutyric, and butyric acids. Further, a mild foot odour was observed in sensory tests in human-being by utilizing cultures of *S. epidermis, C. minutissiumum* and *S. hominis,* amild foot odour was observed. Whereas, in cultures of *Bacillus,S. aureus, P. avidum* and *P. granulosum* a strongfoot odour was found. Bacillus strain such as *B. subtilis* is considered to be participating in enhancing foot odor and was found in cultures having intense foot odour [5]. According to Ara *et al*., leucine dehydrogenase activity was observed in foot skin microflora such as *S. epidermis, genus Propionibacterium, Corynebacterium,* andBacteroides [6]. Via leucine dehydrogenase activity, *Staphylococcus epidermis* metabolizes leucine supplied by secretion of the gland[6,7]. Thus, these studies confirm that *S. epidermis* is accountable for an isovaleric acid generation.

Researchers also noted that by amino acid breakdown, *Propionibacterium* Acnes could generate isovaleric acid and propionic acid in small quantities. Marshall *et al.* [7] observed that the intensity of foot odor generally depends on two factors which include a) enzymes quantity available to damage the skin corneal layer and conversion of sweat ingredients into amino acids, and b) the amount of bacteria present with enzymes required to produce odiferous compounds by decaying amino acids. It can be possibly interpreted that the occurrence of *S. epidermis* and *P. acnes* may be related to isovaleric and propionic acid quantities, which are responsible for odor of feet, whereas genus *Bacillus* bacteria can be responsible for increasing the intensity of the malodor.

The larger population relies on using anti-perspirant or deodorants to eliminate foot odor. The foot deodorants such as powder, sprays, stick etc. are available in the market, which may target the microorganism/s involved in causing foot odour. Most of the available products either use chemical actives such as zeolite, triclosan, or alumina. These products either act against the natural sweating mechanism or develop bacterial tolerance. As an alternative, a few products in the form of a spray containing one or more natural oil, including Tea tree oil, cinnamon oil, peppermint oil, and thyme oil, have been explored and are available as a deodorant. However, these products are

ineffective against bacteria such as *Bacillus subtilis*, *Staphylococcus epidermidis* and *P. acne*. Further, these essential oil-based products have stability issues due to phase separation and produce stains on foot skin or apparel. The present study aims to determine the optimized combination of natural actives which can show synergistic activity against these bacteria and solubilize them in suitable solubilizer/s in order to formulate a liquid roll-on preparation having desired stability and sensory studies.

2. MATERIALS AND METHODS

2.1 Materials

The essential oils such as Neem oil (*Azadirachta indica*), Lemon oil (*Citrus limon*), and Tulsi oil (*Ocimum tenuiflorum*) were procured from the local supplier of Pune, Maharashtra. The excipients such as glycerine, carbopol, hydroxypropyl methylcellulose, ultrez, ethanol, isopropyl alcohol, PEG 40 hydrogenated castor oil, polyglyceryl-3 caprylate /caprate/succinate, propylene glycol,PVP/VA copolymer, HPC, Xanthan gum, Carbopol and triethanolamine were procured from the local supplier of Pune, Maharashtra.

2.2 Culture and growth medium

Bacillus subtilis, and *Staphylococcus epidermidis* strains were obtained from the Department of Microbiology, Modern College of Pharmacy, Pune. Cultures were reserved and developed on Mueller-Hilton broth at 37^{o}C.

2.3 Lethal effect determination against *B. subtilis* and *S. epidermidis*

Oil samples were prepared using a mixture of lemon oil, neem oil and tulsi oil with DMSO (dimethyl sulfoxide, 10%) in M-H broth (Mueller-Hinton broth). 0.5 ml of the sample was mixed with *B. subtilis* or *S. epidermidis* suspension (0.1 ml, 110 CFU ml-1*).* Control was prepared using a mixture of DMSO (10%, 0.5ml) and M-H broth mixed with *B. subtilis* or *S. epidermidis suspension* (0.1 ml, 110 CFU ml-1*).* Sample and control were taken in separate tubes and stirred properly. First, 0.1 ml of sample and control were transferred to separate test tubes containing saline solution (0.9 ml). The transfer of sample or control is carried out at 0 min, 10 min, 20 min, 30 min and 60 min. The obtained samples were further subjected to serial dilution (10-fold) in a saline solution. The sample was then expanse over M-H agar to perform viable counting. The test was performed three times. The obtained results are shown as time-log survivor's curves.

2.4 Solubilization of oils

A mixture of neem oil, tulsi oil and lemon oil was mixed in propylene glycol; ethanol; Isopropyl alcohol and water. These preparations were further optimized using one or more solubilizer/s such as propylene glycol, polysorbate 20, PEG 40 hydrogenated castor oil, polyglyceryl-3 caprylate /caprate/succinate; propylene glycol. The amount of solubilizer was varied and different ratio of two solubilizers were also tried. The obtained preparations were tested for solubility and stability.

2.5 Preparation of Liquid roll-onpreparation

Different preparations in the form of a liquid roll-on were prepared by using one or more polymer selected from Carbopol, xanthan gum, hydroxy propyl cellulose and PVP/VA copolymer**.**
Liquid Roll-on formulations, 1A-1F were prepared using Carbopol, Liquid Roll-on formulations 2A-2F were prepared using Xanthan gum, and Liquid Roll-on formulations 3A-3F were prepared using HPC and PVP/VA copolymer.

2.6 Testing of liquid roll-on

2.6.1 Viscosity

The viscosity of developed liquid roll-onpreparation was tested using a Brookfield digital viscometer. A spindle (no. 6) was inserted into a liquid roll-on sample and rotated at 50 rpm, 27 ± 1°C temperature for 15 min. The reading in triplicate was noted. Viscosity in centipoise (cp) was measured.

2.6.2 pH

1% aq. a solution of liquid roll-onpreparationwas prepared and stored for 2 h. The preparation was then tested for pH using a digital pH meter. The pH of each preparation was determined in triplicate, average value and ± standard deviation were calculated.

2.6.3 Homogeneity

All developed liquid roll-on preparations were allowed to be set in a suitable container and tested for homogeneity by visual inspection, and liquid roll-on appearance was reported.

2.6.4. Skin Irritation testing and Acceptability study

The optimized liquid roll-on was selected for performing skin irritation testing. Twenty volunteers were subjected to testing. A research protocol and possible side effects were shared with the volunteer before signing a consent form. The test was conducted by spreading roll-on (1 ml) on the foot-sole of volunteers, and observations were noted after 5 min. All volunteers were informed

to note the acceptance of liquid roll-on and skin irritation using a form containing predefined questions, including the appearance ofliquid roll-on, odour, texture, redness, and itching post use of the liquid roll-on preparation.

2.6.5 Organoleptic Test

The optimized preparation was examined for appearance and color. In addition, the preparation was also tested for pre and post-application odour.

2.6.6 Physical Stability

A freeze-thaw cycle was used to evaluate the physical stability of the optimized liquid roll-on. The freeze-thaw cycle technique involved storing the sample at 4^0C and 45^0C for 24 h. Two days time frame was utilized for performing one complete cycle. Then, 5 cycles were performed using 10 days. The parameters such as pH, color, odour, pH, homogenity and drying time were noted before the first cycle and after the fifth cycle, post spreading of liquid roll-on.

2.6.7 Determination of antibacterial activity

i) Preparation of inoculums

Fresh bacterial cultures of *Bacillus subtilis*, and *Staphylococcus epidermidis* were separately dispersed in sterile water for 24 hours to get two suspensions of microorganisms.

ii) Preparation of Nutrient Agar Media

Agar 15.0 g, Beef extract 3.0 g and Peptone 5.0 g were accurately weighed and transferred into a conical flask. To this, the required quantity of distilled water was added and stirred the obtained mixture of nutrient agar media for 2 min at the boiling point. Next, the medium was subjected to autoclave sterilization at 121°C for 15 min.

iii) Determination of the zone of inhibition

The antibacterial activity of the liquid roll-on was performed using the agar well diffusion method. This method transferred 15-20 ml of a previously liquefied medium into sterile test tubes. These test tubes were then cooled to 42°C-45°C temperature. One loopful of the culture was transferred into each agar medium containing a test tube and mixed. The obtained inoculated liquid agar medium was then transferred to a separate sterile petri plate subjected to solidification. After solidification of the medium, the required quantity of liquid roll-on preparation was applied to the cavities of the agar plate, and the agar plate was subjected to incubation at 37°C ± 1°C for 24 hours.

2.6.8 Biological stability study of liquid roll-on preparation

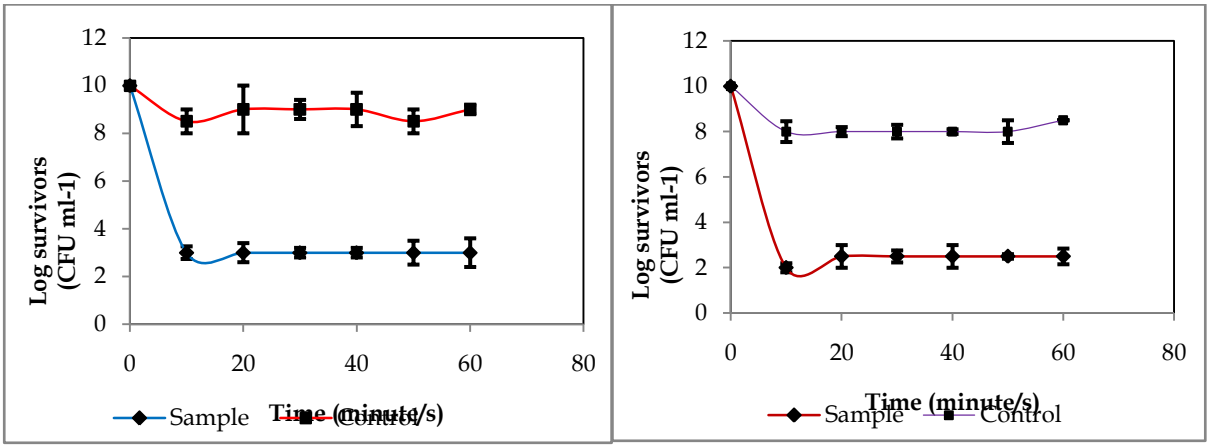
One liquid roll-on sample was stored at room temperature, whereas another sample was stored at 45°C. The lethal effect was determined at various time intervals, e.g. at day 0, on 15th day, on 30th day, and on 60th day to assess the biological stability. First, 0.9 ml of the liquid roll-on preparation and 0.1 mL of bacterial suspension of 110 CFU ml-1 were mixed properly. Next, the obtained samples were reserved at 37°C. Then, 0.1 ml of the sample was subjected to serial dilution (10-fold). The exposure time was 0 and 1 hour. Mueller-Hinton agar was then spread with the sample and incubated for 24 hours. Survival bacteria were counted post-incubation. A log survivor versus time graph was prepared to represent the log reduction of bacteria.

A student t-test was performed to evaluate the statistical difference in the biological stability for foot-liquid roll-on samples stored at room temperature and at 45°C for each of *B. subtilis* and *S. epidermidis*. The p-value was found to be below 0.05.

3. RESULTS

The lethal effect of a mixture of lemon oil, neem oil and tulsi oil against *B. subtilis* was measured to check the efficacy of the combination of three oils for liquid roll-on preparation.

As shown in Figure 1 (A and B), the results clearly indicate that the mixture of lemon oil, neem oil and tulsi oil rapidly reduces *B. subtilis* and *S. epidermidis*population. The initial bacterial count (*B. subtilis* or *S. epidermidis*) which was approximately 110 CFU ml-1 was diminished to 103 CFU ml-1 or less for *B. subtilis* and 102 CFU ml-1 for *S. epidermidis*within 10 minutes after exposing to the mixture of oils. Accordingly, the mixture of oils was found to diminish at least 7 and 6 log of the primary populace of *B. subtilis* and *S. epidermidis*,respectively, in 10 min. Furthermore, the effect was continued for 60 min of exposure.



(A) **(B)**

Figure 1. Time killing curve, Mean ±SD (standard deviation), n=3; **(A)** on *B. Subtilis*; **(B)** on *S. Epidermidis*; CFU: Colony Forming Unit

Different liquid roll-on preparationswere prepared by using one or more polymer selected from Carbopol, xanthan gum, hydroxy propyl cellulose and PVP/VA copolymerand solubilizer/s such as PEG 40 hydrogenated castor oil, polyglyceryl-3 caprylate /caprate/succinate.

Table 1: Liquid Roll-on formulations (1A-1F)

Ingredients	**Quantity (mg/gm)**					
	A	B	C	D	E	F
Lemon oil	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Neem oil	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Tulsi oil	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg
Propylene glycol	36 gm	36 gm	36 gm	36 gm	36 gm	36 gm
PEG 40 hydrogenated castor oil	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm
Polyglyceryl-3 Caprylate /Caprate/Succinate; Propylene Glycol	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm
Ethanol	40 gm	40 gm	40 gm	40 gm	40 gm	40 gm
Isopropyl alcohol	2.2 gm	2.2 gm	2.2 gm	2.2 gm	2.2 gm	2.2 gm
Carbopol	0.3 gm	0.1 gm	0.2 gm	0.4 gm	0.5 gm	1.0 gm
Triethanolamine	0.3 gm	0.3 gm	0.3 gm	0.3 gm	0.3 gm	0.3 gm
Water	Q.S.	Q.S.	Q.S.	Q.S.	Q.S.	Q.S.

Table 2: Liquid Roll-on formulations 2A-2F

Ingredients	**Quantity**					
	A	B	C	D	E	F
Lemon oil	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Neem oil	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Tulsi oil	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg
Glycerin	55 gm	55 gm	55 gm	55 gm	55 gm	55 gm
PEG 40 hydrogenated castor oil	0.25gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm

Polyglyceryl-3 Caprylate /Caprate/Succinate; Propylene Glycol	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm
Ethanol	40 gm	40 gm	40 gm	40 gm	40 gm	40 gm
Isopropyl alcohol	2.2 gm	2.2 gm	2.2 gm	2.2 gm	2.2 gm	2.2 gm
Xanthan gum	0.5 m	0.1 gm	0.2 gm	0.3gm	0.4 gm	0.1 gm
Triethano-lamine	0.3 gm	0.3 gm	0.3 gm	0.3 gm	0.3 gm	0.3 gm
Water	Q.S.	Q.S.	Q.S.	Q.S.	Q.S.	Q.S.

Table 3: Liquid Roll-on formulations 3A-3F

HPC and PVP/VA copolymer

Ingredients	**Quantity (mg/gm)**					
	A	B	C	D	E	F
Lemon oil	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Neem oil	20 mg	20 mg	20 mg	20 mg	20 mg	20 mg
Tulsi oil	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg
Glycerin	1gm	1gm	1gm	1gm	1gm	1gm
PEG 40 hydrogenated castor oil	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm
Polyglyceryl-3 Caprylate /Caprate/Succinate; Propylene Glycol	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm	0.25 gm
Ethanol	64 gm	64 gm	64 gm	64 gm	64 gm	64 gm
IPA	3 gm	3 gm	3 gm	3 gm	3 gm	3 gm
HPC	0.5 gm	0.1 gm	0.3 gm	0.7 gm	0.4 gm	0.6 gm
PVP / VA copolymer	0.1gm	0. 2 gm	0.3 gm	0.4 gm	0.5 gm	0.6 gm
Triethano-lamine	0.3 gm	0.3 gm	0.3 gm	0.3 gm	0.3 gm	0.3 gm
Water	Q.S.	Q.S.	Q.S.	Q.S.	Q.S.	Q.S.

Results of pH, viscosity, stickiness, homogeneity and drying time for formulation 3A are shown in Table 4.

Table 4: Properties of liquid roll-on formulation

Properties	Before 1st Cycle	After 5th Cycle
Appearance	Clear & Transparent	Clear & Transparent
pH	7.1	7.2
Viscosity	651 cps	655 cps
Stickiness	No	No
Homogeneity	Yes	Yes
Drying time	<15 seconds	<15 seconds

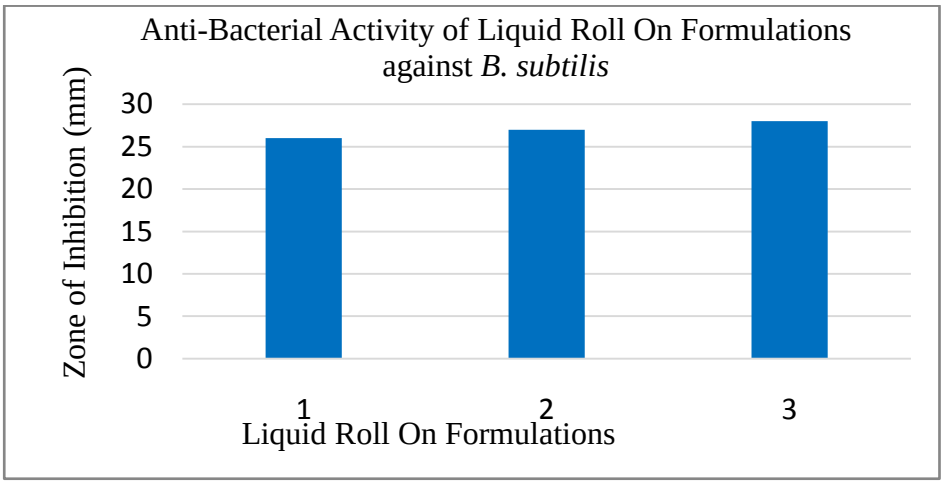


Fig. 2: Anti-Bacterial Activity of Liquid Roll-On Formulations against *B. subtilis*

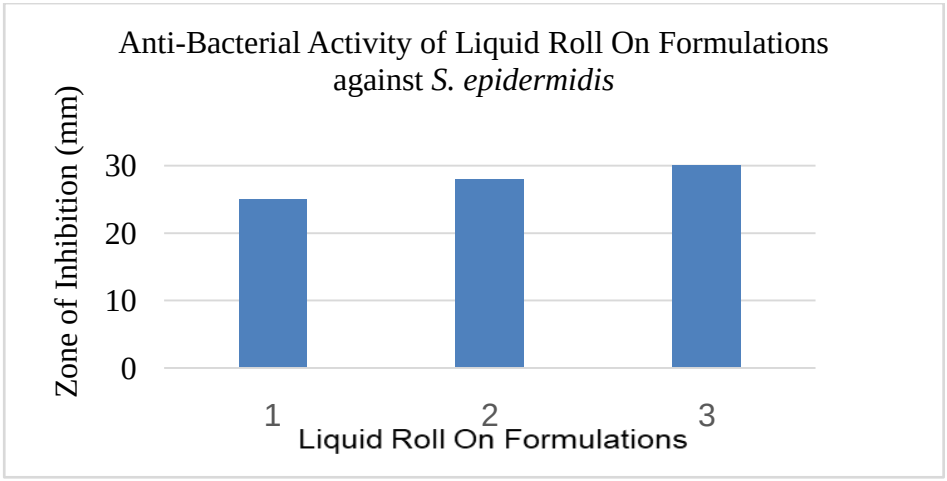


Fig. 3: Anti-Bacterial Activity of Liquid Roll-On Formulations against S. *epidermidis*

The pH value is found to be in the regular pH range of skin. The result of viscosity shown in Table 4 indicates that the prepared roll-on preparation was of optimum viscosity, which can satisfy ease of application of delivery on skin.

. The selected liquid roll-on preparation3Awas found to have desired consistency/homogeneity and was devoid of any lumps.

The liquid roll-on containing lemon oil, neem oil and tulsi oil was chosen to perform acceptability and skin irritation. The liquid-rollexhibited good spreadability with no stickiness, comfort post application, and outstanding antibacterial and deodorizing effect. The liquid roll-on was very well-accepted when tested for skin irritation test. None of the volunteers reported any symptoms of itching or redness.

The organoleptic test of the liquid roll-on was performed to assess the physical appearance of the liquid roll-on preparation. The results show that the liquid roll-onexhibit a homogenous appearance, pleasing odor, and constant flow. Further, the liquid roll-on was found to be easy to spread upon application. The liquid roll-onalso showed homogeneity when spread on glass, and no coarse particles were visible.

The physical stability of the liquid roll-on was checked using a freeze-thaw cycling method. The results in Table No. 4 indicated that the liquid roll-onwas found to have desired physical stability. In addition, the properties of liquid roll-on preparation, such as appearance, stickiness, viscosity, drying time and homogeneity, did not change after freeze-thaw cycling storage. The pH was also found to be constant.

When exposed to liquid roll-on, the bacteria, namely, *B. subtilis* and *S. epidermidis*, were reduced to 90% or more (1 log) when checked for log reduction. The result shows that the liquid roll-ontested at 450C exhibits a bacterial reduction ability that is not statistically significant ($p>0.05$) compared to room temperature.

The antibacterial activity of the given liquid roll-onis provided in Figures2and 3. Amongst various formulations (1A,2A and 3A), the liquid roll-on3A show the higher zone of inhibition against both the bacteria, namely *Bacillus subtilis* and *Staphylococcus epidermidis,* compared to liquid roll-on preparations 1A and 2A.

4. DISCUSSION

Plant essential oils are known for the treatment and/or prevention of various infections or diseases as an alternative to allopathy medicines due to their no or fewer side effects. Various essential oils have been tested in past by various researchers against bacterial pathogens. The

phytoconstituent/s present in oils is effective against these pathogens. Hydrophobicity of oil may help enter into the bacterial cell membrane's lipids, thereby changing the cell structure and making it pervious which finally causes cell death. Amongst various essential oils, lemon oil has been reported to have antibacterial activity against various microbes. The antibacterial activity of lemon oil against these bacteria may be attributed to limonene, alfa-pinene sabinene, carene, and β-ocimene [8,16].Neem extract has been reported to have activity against *S. Epidermidis*[17-22]. Furthermore, Tusli leaves extract's activity against *B. subtilis* has also been reported. Tulsi leaves (*Ocimum sanctum* L.) have active secondary metabolite compounds that act as antibacterials. Tulsi leaves (*Ocimum sanctum* L.) also contain high linolenic acid, which functions as antibacterial activity[23-31].

In the present research, the liquid roll-on preparation containing a combination of lemon oil, neem oil and tulsi oil was found to have the best activity against *B. subtilis* and *S. epidermidis,* which are generally found in the planter skin of human-being who has strong foot odour. The effiicacy of the liquid roll-onwas linked with the stability of the product which is achieved by using a combination of two solubilizers, namely PEG 40 hydrogenated castor oil and Polyglyceryl-3 Caprylate /Caprate/Succinate; propylene glycol.

The acceptability and efficacy of liquid roll-on preparation mandate the formulation to hold ideal physicochemical characteristics, such as viscosity, easy application, ease of removal from the storing means and antibacterial action against odor-producing bacteria. The liquid roll-on preparation3A exhibited a pH value in the usual pH range of the skin and was found to be non-harmful. The liquid roll-on preparation didn't produce any stain on the skin surface of the subject as well as on the socks or cloth in contact with the foot. The effectiveness of a liquid roll-on preparationmay also rely upon spreading capacity. The spreadability of the liquid roll-on preparation was found to be optimum. The viscosity of the liquid roll-on preparationis an important feature in judging the possible mucoadhesive capacity of the liquid roll-on preparation. The liquid roll-on preparationshowed appropriate viscosity at different shear rates. The liquid roll-on preparationexhibits significant stability in terms of biological activity as it can reduce at least 1 log, i.e. 90% of *B. subtilis* and *S. epidermidis* populace over 60 days.

5. CONCLUSION

Liquid roll-on preparation containing a combination of lemon oil, neem oil and tulsi oil (Formulation 3A) showed a greater zone of inhibition against *B. subtilis* and *S. epidermidis* respectively than other liquid roll-on preparations(liquid roll-on preparation1A and 2A). Thus, it

was observed that the optimized liquid roll-on preparationwas more effective against *Bacillus subtilis*, and *Staphylococcus epidermidis* and can be used as an effective foot deodorant. The liquid roll-on preparation showed the ease of application and reduced foot odour with no skin itching or irritation. Accordingly, the liquid roll-on preparation can be used by people with high sweating problems, who frequently engage in sports/exercise involving high exertion, or by people with strong foot odour. In addition, the developed liquid roll-on preparation can reduce *Bacillus subtilis*, and *Staphylococcus epidermidis* over a longer period under accelerated conditions. More clinical investigations on human are needed to check deodorizing efficacy of the present liquid roll-on preparation to confirm the present finding.

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