Annals of Phytomedicine 3(1): 37-42, 2014

Journal homepage: www.ukaazpublications.com

ANNALS OF PHYTOMEDICINE
An International Journal

ISSN 2278-9839

Antimicrobial potential of topical formulation containing essential oil of *Eucalyptus citriodora* Hook.

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Received May 5, 2014: Revised June 10, 2014: Accepted June 15, 2014: Published online June 30, 2014

Abstract

Topical formulations containing essential oil of *Eucalyptus citriodora* Hook. (2%) were developed for their promising antimicrobial activity against selected pathogenic bacteria and fungi. The formulations (cream and ointment) were prepared using standard methods and assessed for different pharmaceutical parameters. *In vitro* antimicrobial studies of the formulations and essential oil were performed by using agar disc diffusion and agar well diffusion techniques. Ointment formulation showed broader range for zone of growth inhibition (ZGI; 10.0 ± 0.58 to 27.0 ± 1.15 mm) compared to cream formulation (ZGI; 5.0 ± 0.57 to 19.67 ± 0.88 mm) against selected strains of microorganisms. Texture parameters of ointment formulation in terms of firmness (82.89 ± 0.38 g), spreadability (6.13± 0.06 mJ), extrudability (544.00 ± 3.73 mJ), adhesiveness (3.07 ± 0.12 g) and cohesiveness (0.92 ± 0.01) were evaluated using CT3 texture analyser. Data obtained in the form of zone of growth inhibition (mm in diameter) indicate that the activity of ointment formulation was more pronounced against *Sporothrix schenckii, Candida albicans*, *Mycobacterium smegmatis* and *Salmonella typhi*. The results of efficacy and aesthetic appearance vouch that consistent and pharmaceutically elegant cream and ointment formulations of eucalyptus oil have been developed with potent antimicrobial activity.

Key Words: *Eucalyptus citriodora* Hook., essential oil, antimicrobial, cream formulation, ointment formulation, texture analysis

1. Introduction

Infectious diseases represent prominent health issues in both developed and developing nations with an alarming increase in the incidence of new and emerging drug resistant microbes due to indiscriminate use of antibiotics and other drugs (Viswanad *et al*., 2012; Gilani *et al*., 2013). To combat the challenge of multi drug resistance pathogens, scientists, researchers and pharmaceutical industries have been looking into natural products derived from plants as an alternative to the synthetic counter parts (Gandomi *et al*., 2013; Jinukuti and Giri, 2013). Essential oils obtained from aromatic plants, spices, and herbs are being investigated as a potential source of novel antimicrobial agents possessing broad spectrum antimicrobial activities. They are not only found effective in the treatment of infectious diseases but also mitigate many of the side effects that are often associated with synthetic antimicrobial agents (Hemlatha *et al*., 2013; Tarek *et al*., 2014).

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The essential oil of *Eucalyptus citriodora* Hook. (Myrtaceae) is widely known for its perfuming and flavouring potential and has been introduced in India for reclamation of waste lands, timber and wood production as well as for pulp, fuel and volatile oil (Ali *et al.,* 2014). The oil is traditionally used for its number of medicinal properties including potent antiseptic action, to cure cuts and skin infections, inhaled to open blocked nasal passages as a respiratory decongestant as well as for relieving colds, coughs, bronchitis, flu, pneumonia and headache, gargles for sore throats and also as a natural pesticide (Vaghasiya *et al.,* 2008; Batish *et al.,* 2008). The resin of *E. citriodora* has been employed in the treatment of diarrhoea and bladder inflammation. The oil possesses remarkable analgesic, anti-inflammatory (Silva *et al.,* 2003), antioxidant (Singh *et al.,* 2012), antimicrobial (Luqman *et al.,* 2008), antibacterial (Cimanga *et al.,* 2002; Nair *et al.,* 2008), antifungal (Ramezani *et al.,* 2002; Javed *et al.,* 2012), antituberculosis (Alvarenga *et al.,* 2014), larvicidal, acaricidal (Goerge *et al.,* 2009; Clemente *et al.,* 2010), anticandidial (Dutta *et al.,* 2007), cytotoxic, antitumor, allelopathic, insect repellent (Olivero-Verbel *et al*., 2010), insecticidal effects on *Lutzomyia longipalpis* (Maceil *et al.,* 2010), antiproliferative (Duh *et al.,* 2012), fumigant (Han *et al.,* 2011), anthelmintic (Macedo *et al.,* 2011), diuretic, and antispasmodic activities. The glabrous leaves of *E. citriodora* contain essential oil with 65.5% citronellal while hairy leaves as much as 86-90% which gives it a wonderful lemony aroma. Other compounds present in it

include geranyl acetane, phenylethyl phenylacetane, limonene, terpinen-4-ol and torquatone (Vaghasiya *et al.,* 2008). The leaves also contain tannins, phenolics, flavonoids like eucalyptin, hyperin, hyperoside, quercetin, quercitrin, rutin, sesquiterpenes, aldehydes and ketones (Kharwar *et al.,* 2010).

The potent antimicrobial properties of *Eucalyptus citriodora* oil (ECO) have already been reported, therefore, the present study takes into consideration the possible use of ECO as a natural antimicrobial agent by means of a suitable formulation. With this aim, topical cream (CF) and ointment formulations (OF) containing ECO were prepared and evaluated for their pharmaceutical parameters. The developed formulations were also investigated for their *in vitro* antimicrobial activity against selected pathogenic strains of topical microbial flora.

2. Materials and Methods

2.1 Materials

Essential oil of *Eucalyptus citriodora* Hook. was procured from Process Chemistry and Chemical Engineering Department, CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India. The oil was analyzed by gas chromatography on Nucon Equipment (New Delhi, India). Light liquid paraffin was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. Soft white paraffin and paraffin wax were procured from SD Fine Chem Limited, Mumbai, India. Olive oil, cetyl alcohol, stearyl alcohol, glycerol monostereate and sodium lauryl sulphate were supplied from CDH Ltd., New Delhi, India and polyethylene glycols were procured from Thomas Baker Chemicals Pvt. Ltd., India. All the chemicals used in the study were of analytical grade and double distilled water was used throughout the study.

2.2 Microorganisms used

The following pathogenic strains were used for the study: *Staphylococcus aureus* MTCC-96, *Mycobacterium smegmatis* ATCC-10231, *Salmonella typhi* MTCC-733, *Staphylococcus aureus* ATCC, *Salmonella typhimurium* MTCC-98 (all bacteria) and *Cryptococcus neoformans* MTCC-1436, *Sporothrix schenckii* MTCC- 1359, *Candida albicans* AIIMS, C*andida albicans* ATCC (all fungi).

2.3 Preparation of inoculum

Cultures of bacteria and fungi (24 and 48 h) were grown on Nutrient and Sabouraud Dextrose Media (Himedia Pvt, Ltd., New Delhi, India) respectively and the turbidity was compared with that of 0.5 McFarland standards (approximately 1-2 x 10^8 CFU/mL). They were then incubated overnight (16-24 h) in the respective broth media at 37°C and 28°C for the bacteria and fungi, respectively. The working inoculums were prepared by suspending one loop full of pathogenic microbes in one millilitre of autoclaved sodium chloride (0.85%).

2.4 Preparation of topical formulation

The ECO (2%) was incorporated into CF and OF formulations for its antimicrobial activity evaluation. The formulations containing ECO were prepared using standard methods. Phase inversion temperature technique was employed to formulate the cream with the excipients listed in Table 1. The ingredients of oil phase (phase A) and aqueous phase (phase B) were heated (50-60°C) in separate beakers and stirred on magnetic stirrer (IKA, India). Then, both the phases were mixed with constant stirring for proper emulsification. When the system attained a temperature of 40°C essential oil was incorporated in the mixture, resulting in white coloured smooth CF. Hydrophilic ointment (OF) was prepared by incorporating the essential oil in heat melted macrogol blend under constant stirring at 40°C. The composition of OF is indicated in Table 2. Placebo formulations were also prepared for comparative study. Both the formulations were stored in properly labelled collapsible tubes and kept at room temperature for further evaluation.

Table 1: Composition of cream formulation (CF)

Ingredients	Percent concentration
Phase A	
Light liquid paraffin	6.0
Soft white paraffin	2.0
Olive oil	1.0
Paraffin wax	2.0
Cetyl alcohol	3.0
Stearyl alcohol	3.0
Glycerol monostereate	3.15
Methyl paraben	0.1
Essential oil	2.0
Phase B	
Propyl paraben	0.05
Sodium lauryl sulphate	0.85
Purified water	q. s.

Table 2: Composition of ointment formulation (OF)

Ingredients	Percent concentration
PEG-4000	35.0
PEG-400	50.0
Essential oil	2.0
Propyl paraben	0.08
Distilled water	q. s.

2.5 Pharmaceutical evaluation of formulations

2.5.1 *Drug content measurement*

Specified quantity of weighed CF and OF was dissolved in 100 ml distilled water and measured spectrophotometrically using UV-Visible Spectrophotometer (Shimadzu 1800) at 232 nm. Experiments were performed in triplicate.

2.5.2 *pH measurement*

About 10% (w/v) solution of CF and OF in distilled water was prepared to measure the pH using a digital pH meter (Mettler Toledo, Model LE-438) at 25 ± 1° C. Experiments were performed in triplicate.

2.5.3 *Viscosity measurement*

Viscosity of both the formulations CF and OF was measured using Brookfield Viscometer DVLV-II + Pro Model at 25 ± 1°C and 0.5 rpm speed by Spindle No. 96. Measurements were performed in triplicate.

2.5.4 *Texture analysis*

CF and OF were also evaluated for different texture parameters depicting human sensory perceptions namely, firmness, spreadability, extrudability, adhesiveness and cohesiveness by using CT3 Texture Analyzer (Brookfield Engineering Laboratories, USA). All the graphs and data were generated using Texture Pro CT V1.3 Software, and formulations were evaluated in triplicate.

2.6 Antimicrobial activity

The antimicrobial activity of the ECO (positive control) was determined by agar disc diffusion method, whereas, well diffusion method was adopted for prepared topical formulations. 100 µL of inoculum of the respective microbial culture was withdrawn with caution and spread uniformly over the surface of agar plate to get an even lawn. In disc diffusion method, 5 µL of oil was impregnated on the sterile paper disc (5 mm diameter, Whatman filter paper) and placed on the premade bacterial and fungal lawns. The plates were then incubated for 24 h (37°C) and 48 h (28°C) for bacteria and fungi, respectively. The zone of growth inhibition was measured in millimeters (mm) and values are reported as mean of three experiments in replicate. CF and OF were evaluated by well diffusion technique by making equidistant wells of even size (7mm diameter each) with the help of sterilized borer. Equal sample of formulations and blank was poured into the wells and incubated 24 h for bacterial strains at 37°C and 48 h for the fungal strains at 28°C. All plates were examined for zone of growth inhibition and their diameters were measured. The values reported are mean of three experiments.

3. Results and Discussion

3.1 Pharmaceutical evaluation of formulations

ECO was formulated into cream and ointment which do not show considerable changes in characters like colour, odour, consistency and there was no phase separation, observed during the course of the study. The prepared formulations were also evaluated for their pH, viscosity, drug content and textural sensory evaluations which depicted the human sensorial parameters like hardness, spreadability, extrudability, adhesiveness and cohesiveness.

3.1.1 *Drug content*

Drug content of CF and OF was found to be 89.03 ± 0.25% and 92.42 ± 0.30 %, respectively, as tabulated in Table 3.

3.1.2 *pH measurement*

The pH values of 10 % solution of the developed CF and OF were 5.83 ± 0.03 and 6.07 ± 0.02, respectively. The result shows that the formulations are considered acceptable without the risk of any irritation on application to the skin.

3.1.3 *Viscosity measurement*

The viscosity of the both the formulations CF and OF was measured by a Brookfield viscometer using Spindle No. 96 at 0.5 rpm at room temperature and was found to be 1086388 ± 12.01 cps and 2995683 ± 14.53 cps, respectively. The values are represented in Table 3. In topical formulations, consistency is a substantial factor affecting spreadability and overall acceptance of the formulation. The apparent viscosity at this low shear rate is an indicator of spreadability upon topical application (Sahin *et al*., 2011). Also, according to the Stoke's law, viscosity is inversely proportional to sedimentation/creaming, thereby, affecting the stability of the formulation (Yadav *et al.,* 2013).

3.1.4 *Texture analysis*

Human sensorial perception in terms of firmness, spreadability, extrudability, adhesiveness and cohesiveness, collectively called texture parameters, were depicted for CF and OF, using particular probes through CT3 Texture Analyzer (Brookfield Engineering Laboratories, USA) (Yadav *et al*., 2013; Rai *et al*., 2014). These parameters do not have any fixed selection criteria or any official standards (pharmacopoeial/regulatory), but based on the product specific requirement, these parameters can be characterized and standardized (Meher *et al.,* 2012). The cream and ointment formulations were compared on the basis of values obtained for each parameter as recorded in Table 3.

Table 3: Pharmaceutical evaluation of cream (CF) and ointment formulation (OF)

		Cream formulation	Ointment formulation
pH		5.83 ± 0.03	6.07 ± 0.02
Viscosity (cp)		1086388 ± 12.01	2995683 ± 14.53
Drug content (%)		89.03 ± 0.25	92.42 ± 0.30
Texture profile	Firmness (g)	37.27 ± 0.38	82.89 ± 0.38
	Spreadability (mJ)	3.87 ± 0.03	6.13 ± 0.06
	Adhesiveness (g)	3.71 ± 0.13	3.07 ± 0.12
	Cohesiveness	0.97 ± 0.01	0.92 ± 0.00
	Extrudability (mJ)	130.51 ± 1.49	544.00 ± 3.73

The values are expressed as Mean ± SEM, n=3

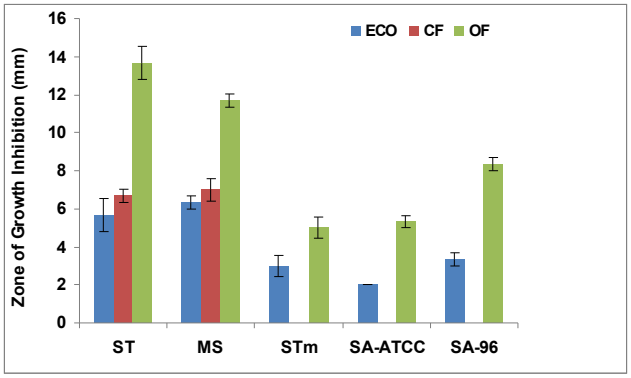
The maximum positive force required to deform the sample, *i.e.*, the formulation with the finger imitated by the probe is the measure of the firmness of that sample. As clear from the values, OF required more force (82.89 ± 0.38 g), thus, showing OF harder than CF (37.27 ± 0.38 g), which may be due to the presence of high concentration of macrogols in OF. Spreadability is another noteworthy parameter affecting overall elegance and consumer's acceptance to the topical formulation. It is defined as the amount of work required for spreading the sample over a surface. CF was found to be more spreadable (3.87 ± 0.03 mJ) than OF (6.13 ± 0.06 mJ), which also supports the findings of firmness. The formulation which is more spreadable has lower firmness value (Inoue *et al.,* 2012). Similarly, extrudability is the force required to uniformly extrude the sample out of its container. A formulation with optimum spreadability and extrudability is easily acceptable due to its user friendly aspects. The work done to extrude CF from the tube (130.51 ± 1.49 mJ), was comparably less than that required for OF (544.00 ± 3.73 mJ), showing better extrudability of CF from its respective tube. Poor and nonuniform extrudability is indicative of deterioration or instability of any particular formulation as well as any sort of incompatibity if persists, between sample and its container. As a quantitative parameter of the stickiness of the sample, adhesiveness is considered as the maximum force required for overcoming the attractive force between any surface and sample (cream). CF required 3.71 ± 0.13 g force to separate from its surface, where as OF 3.07 ± 0.12 g. Comparatively, CF required higher force than OF in adhesiveness test; hence, it was found to be more adhesive.

Cohesiveness is another parameter which indicates the strength of internal bonds that is responsible for overall elegance of the formulation. Mathematically it is the ratio of hardness work done at two cycles. Both CF and OF exhibited similar values for cohesiveness.

3.2 Antimicrobial activity

The essential oil and its topical formulations were tested against selected pathogenic strains of bacteria and fungi. Before determining the activity of the formulations, the antimicrobial activity of ECO was assayed *in vitro* by agar disc diffusion method against the selected microbes. The results of the antimicrobial study are summarized in Figures 1 and 2. ECO was active against all the pathogenic bacteria and fungi under study as revealed by their respective zones of growth inhibition. No growth inhibition was observed in case of blank formulations. However, the formulations displayed a variable degree of antimicrobial activity against the tested strains. As shown in Figure 2, CF containing ECO (2%) was found to be most effective against *Sporothrix schenckii*, displaying the zone of growth inhibition of 19.67 ± 0.88 mm which was found to be more than that of ECO (6.67 ± 0.33 mm). CF also inhibited *Cryptococcus neoformans* and *Candida albicans* (AI and ATCC) with inhibition zones of 5.0 ± 0.58 mm, 5.0 ± 0.00 mm and 7.33 ± 0.88 mm, respectively. *Staphylococcus aureus* and *Salmonella typhimurium* were the most resistant bacterial strains with no zones of growth inhibition (Figure 1).

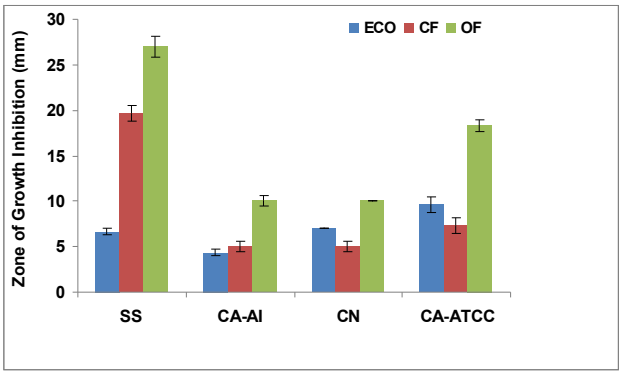
Figure 1: Growth inhibitory activity of *Eucalyptus citriodora* essential oil (ECO), Cream Formulation (CF) and Ointment Formulation (OF) against pathogenic bacteria



SA= *Staphylococcus aureus* MTCC-96; MS = *Mycobacterium smegmatis* ATCC-10231; ST = *Salmonella typhi* MTCC-733; BS = *Bacillus subtilis* MTCC-121; SA-ATCC = *Staphylococcus aureus* ATCC; STm = *Salmonella typhimurium* MTCC-98

The ointment formulation had four times greater zone of growth inhibition to ECO against *Sporothrix schenckii* (27.0 ± 1.15 mm) as depicted in Figure 2. Furthermore, it also effectively inhibited *Candida albicans* (AI and ATCC) and *Cryptococcus neoformans* with the zones of growth inhibition of 10.0 ± 0.58 mm, 18.33 ± 0.67 mm and 10.0 ± 0.00 mm, respectively. In addition, it was also found to be active against *Staphylococcus aureus* and *Salmonella typhi* followed by *Mycobacterium smegmatis* and *Salmonella typhimurium* with an augmentation in activities. The OF was found to illustrate superior antimicrobial activity than CF against all the pathogenic strains.

Figure 2: Growth inhibitory activity of *Eucalyptus citriodora* essential oil (ECO), Cream Formulation (CF) and Ointment Formulation (OF) against pathogenic fungi.



CN = *Cryptococcus neoformans* MTCC- 1436; SS = *Sporothrix schenckii* MTCC- 1359; CA (AI) = *Candida albicans* AIIMS; CA (ATCC) = *Candida albicans* (ATCC)

Antimicrobial activity of ECO can be justified due to its main components, citronellal (60.66%), followed by β-citronellol (12.58%) and isopulegol (8.19%). It has also been proved that the antibacterial activity of *E. citriodora* essential oil is due to synergy between citronellol and citronellal (Cimanga *et al.,* 2002). The oil contains 94.35% of monoterpenes and in addition, significant quantities of β-caryophyllene, p-menthane-3, 8-diol, citronellyl acetate and 1, 8-cineole has also been reported (Singh *et al.,* 2012). These compounds are expected to make a significant contribution towards antimicrobial activity of the essential oil. It was evident from the data that both the formulations were more active towards fungi followed by bacteria, in accordance with the earlier published results (Luqman *et al.,* 2008). It has already been established that active components of essential oils act on various targets of cell employing different mechanisms (Burt, 2004). Phenolic and acetate moieties in essential oil also optimistically influence the antimicrobial actions like EC-OH groups and an allylic side chain enhances the efficacy of essential oil, *e.g.*, geranyl acetate has been found to be more active than geraniol. The mechanism involved includes membrane disruption by lipophilic compounds resulting in inhibition of electron transport, protein translocation, phosphorylation, leakage of contents out of the cell, coagulation of cytoplasm damage to cytoplasmic membrane, depletion of the proton motive force and membrane proteins which ultimately destroy the cell membrane integrity resulting in the death of microorganisms (Gyawali *et al.,* 2014; Jinukuti *et al.,* 2013; Luqman *et al*., 2007; Luqman *et al*., 2008; Burt, 2004).

Interestingly, the enhancement of the antimicrobial activity of ECO in OF could be attributed to better diffusion from hydrophilic ointment base that effectively delivered the ECO to generously proportioned distances. Results revealed that the antimicrobial efficacy of the topical preparations of *E. citriodora* oil was best demonstrated when delivered in the form of a hydrophilic ointment in comparison to CF, entailing that the active moieties in the oil were more readily diffused through a hydrophilic ointment base (Yadav *et al.,* 2012). With an increase in the lipophilic character of the formulation base, a significant reduction in the release of antimicrobial compounds was noted.

4. Conclusion

Topical drug delivery system holds tremendous potential for the delivery of antimicrobial agents prioritizing end user's acceptance and compliance. This study has established an elegant and stable ointment formulation incorporating *E. citriodora* oil, which is active towards both bacteria and fungi. Combination of macrogols used in the formulation of ointment eventually contributes in the efficient diffusion of the active molecules within the media, imparting prominent antifungal and antibacterial activity in comparison to pure oil and cream formulation. Texture parameters articulated the firmness, extrudability, spreadability as well as adhesion capacity revealing the overall elegance and aesthetic appearance of the formulation.

Acknowledgement

The authors are thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi, and Director of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India, for providing financial support and infrastructure facilities under CSIR Network Project ChemBio (BSC 203) for the completion of the present work.

Conflict of interest

We declare that we have no conflict of interest.

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