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Research paper

Microencapsulation of citronella oil for mosquito-repellent application: Formulation and *in vitro* permeation studies

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ABSTRACT

Citronella oil (CO) has been reported to possess a mosquito-repellent action. However, its application in topical preparations is limited due to its rapid volatility. The objective of this study was therefore to reduce the rate of evaporation of the oil via microencapsulation. Microcapsules (MCs) were prepared using gelatin simple coacervation method and sodium sulfate (20%) as a coacervating agent. The MCs were hardened with a cross-linking agent, formaldehyde (37%). The effects of three variables, stirring rate, oil loading and the amount of cross-linking agent, on encapsulation efficiency (EE, %) were studied. Response surface methodology was employed to optimize the EE (%), and a polynomial regression model equation was generated. The effect of the amount of cross-linker was insignificant on EE (%). The response surface plot constructed for the polynomial equation provided an optimum area. The MCs under the optimized conditions provided EE of 60%. The optimized MCs were observed to have a sustained *in vitro* release profile (70% of the content was released at the 10th hour of the study) with minimum initial burst effect. Topical formulations of the microencapsulated oil and non-microencapsulated oil were prepared with different bases, white petrolatum, wool wax alcohol, hydrophilic ointment (USP) and PEG ointment (USP). *In vitro* membrane permeation of CO from the ointments was evaluated in Franz diffusion cells using cellulose acetate membrane at 32 °C, with the receptor compartment containing a water–ethanol solution (50:50). The receptor phase samples were analyzed with GC/MS, using citronellal as a reference standard. The results showed that microencapsulation decreased membrane permeation of the CO by at least 50%. The amount of CO permeated was dependent on the type of ointment base used; PEG base exhibited the highest degree of release. Therefore, microencapsulation reduces membrane permeation of CO while maintaining a constant supply of the oil.

1. Introduction

Over two billion people, primarily in tropical countries, are at risk from mosquito-borne diseases, such as dengue hemorrhagic fever, malaria and filariasis [1]. Malaria, in particular, continues to impart a major disease burden. Nearly 90% of mortality attributed to it is experienced by infants and young children [2]. Mosquito control and personal protection from mosquito bites are currently the most important measures to control this disease [1,3]. The use of mosquito repellents on exposed skin is, therefore, strongly recommended [4,5]. As a result, it has already been accepted as part of an overall integrated mosquito-borne disease control program [6]. In fact, in many circumstances, applying mosquito repellents to the skin may be the only feasible way to protect against mosquito bites [7].

There are a number of effective mosquito repellents containing synthetic chemicals such as N,N-diethyl-3-methylbenzamide, formerly known as diethyl-*m*-toluamide (DEET), and picaridin [8]. DEET is currently the most effective mosquito repellent and is available in various commercial formulations [6]. However, several researchers have reported adverse effects after use of mosquito repellents containing DEET. These include contact urticaria, skin eruption or toxic encephalopathy in children [8–10]. In addition, synthetic chemicals used for control of vectors are causing irreversible damage to the ecosystem, as some of them are non-degradable in nature [6,10]. These problems have highlighted the need for the development of effective non-DEET alternatives. Thus, plant essential oils, commonly used as fragrances and flavoring agents, are recommended as mosquito repellents. Essential oils can be applied to humans in a similar way to other conventional mosquito repellents and they tend to be selective and have little or no harmful effects [6].

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The promising essential oils with mosquito-repellent activity are derived from a large number of plants including *Conyza newii*, *Cympobogon* spp., *Eucalyptus citriodora*, *Lippia javanica*, *Lippia ukambensis*, *Mentha piperita*, *Ocimum* spp., *Pelargonium citrosum*, *Plectranthus marrubioides*, *Tarchonanthus camphoratus*, *Tetradenia riparia*, *Zanthoxylum limonella*, etc. [1]. Especially citronella oil (CO), the essential oil from *Cympobogon* spp., is popular in mosquito-repellent formulations. Candles and incense containing CO are sold as insect repellents in some countries like the United States. Despite the popular conception, it has been reported that citronella candles or incense were ineffective for reducing the biting pressure of mosquitoes [7,9,11]. The possible reasons could be uncontrolled release of the essential oil, i.e., either it is released in too little amount and become ineffective, or it is released excessively with shorter duration of protection. Therefore, how to control the release of these essential oils is a key area worth investigating [12].

Utilizing microencapsulation technology to achieve this goal of controlled release is one of the most effective methods thus far [13–15]. However, at present, there is inadequate study and research related to the technology to encapsulate volatile materials in microcapsules [12]. Thus, the main aim of this study is to encapsulate CO obtained from *Cymbopogon nardus via* simple coacervation microencapsulation technique so as to sustain its *in vitro* release for at least 8 h. To determine the optimal conditions for microencapsulation, the effects of three variables, i.e., stirring rate, the ratio of coating material (gelatin) to core material (CO) and amount of cross-linking agent, formaldehyde (37%), on the encapsulation efficiency (EE, %) were investigated and analyzed by employing response surface methodology. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model that describes the processes fully [16,17]. Details are reported herein. Also, for optimum effect, various semisolid preparations containing microencapsulated essential oil extracts of *C. nardus* were prepared, and their permeations have been investigated *in vitro* using a model membrane.

2. Materials and methods

2.1. Materials

Gelatin (Uni – Chem. reagents, China), sodium sulfate (Uni – Chem. Reagents, China), chloroform (Fluka, Deisenhofen, Germany), geraniol (Fluka, Deisenhofen, Germany), citronellal (Sigma Aldrich, Steinheim, Germany), formaldehyde (Fluka, Deisenhofen, Germany), ethanol (Carl Roth, Karlsruhe, Germany), white petrolatum (Bombastus Werke, Freital, Germany), wool wax alcohol (Bombastus Werke, Freital, Germany), hydrophilic ointment (Bombastus Werke, Freital, Germany), polyethylene glycol (PEG) 400 (Serva Feinbiochemica, Heidelberg, Germany), PEG 4000 (Serva Feinbiochemica, Heidelberg, Germany), isopropyl myristate (Caesar & Loretz, Hilden, Germany) were used as received.

2.2. Essential oil

CO was obtained by steam distillation of the leaves of *C. nardus* at Wondogenet Essential Oil Research Center (Wondogenet, Ethiopia).

2.3. Encapsulation procedure

For encapsulation of CO, simple coacervation technique was employed [18]. In this, an aqueous dispersion containing 10% w/w of gelatin in purified water was prepared at 50 °C, a temperature well below the flash point (70 °C) and the boiling points (200 °C) of most of the components of the essential oil [19]. A specified amount of CO was then added and emulsified with stirring using a magnetic hot plate stirrer (Heidolph MR 3001, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). Coacervation was achieved by gradual addition of an aqueous sodium sulfate solution (20% w/v) at a feeding rate of about 2.5 ml min^{-1}. The resulting mixture was then stirred for 1 h (total coacervation time) to ensure complete deposition of the gelatin onto the oil. The temperature of the system was then brought down to about 5 °C within 1 h, on an ice bath. Formaldehyde solution (37% v/v) was then added to rigidize the gelatin coating. Cross-linking time was set to be 1 h. Finally, the MCs collected were washed three times with ethanol, followed by cold water (5 °C), filtered and dried by lyophilization (Alpha 2-4, Martin Christ GmbH, Osterode am Harz, Germany).

2.4. Gas chromatography/mass spectrometry (GC/MS) analysis

The GC/MS chromatogram of CO was obtained using GC–MS (Finnigan Magnum™, Darmstadt, Germany) equipped with a fused silica capillary column (30 m × 0.25 mm) coated with Optima® – FFAP having a film thickness of 0.25 μm. Helium was used as a carrier gas at a rate of 1 ml min^{-1}. The oven temperature was maintained at 60 °C for 1 min and then increased to 200 °C at a rate of 100 °C min^{-1}. The injector and detector temperatures were maintained at 240 °C. Analysis was done by MS (ion trap temperature at 240 °C; manifold temperature at 220 °C, transfer line temperature at 240 °C).

2.4.1. Construction of calibration curve

A stock solution of 2 mg ml^{-1} of citronellal standard solution was prepared in chloroform. Working solutions were prepared by diluting the stock solution with the same solvent to contain 1, 2, 5, 10, 20, and 40 μg ml^{-1}. Each solution was prepared three times, and triplicate samples of each solution were injected into the GC/MS system; the concentration of CO against peak area was obtained for each sample.

2.5. Encapsulation efficiency

The EE percentage of CO was determined using the method described elsewhere [14]. Accurately weighed amounts of the microcapsules were dispersed in a known volume of chloroform. This dispersion was agitated at 700 rpm for 12 h and then ultrasonic-treated twice for 20 min with 10-min interval. After filtration, using 0.45-μm PTFE filter, quantitative analysis of the supernatant was done by GC/MS. Each experiment was carried out in triplicate. The EE (%) was determined using Eq. (1) [14].

$$\text{EE}\ (\%) = M_a/M_{th} \times 100 \quad (1)$$

where M_a is the actual amount of CO entrapped in the microcapsules and M_{th} is the theoretical amount of CO entrapped in the microcapsules.

2.6. Experimental designs for response surface methodology

A response surface methodology was employed to produce controlled release microcapsules of CO using a three-factor-two-level full factorial design. The stirring rate (X_1, rpm), oil-to-gelatin ratio (X_2) and the amount of formaldehyde (X_3, ml) were the independent variables analyzed. The dependent variable investigated was EE (%). The selected factor combinations indicating the actual and coded levels as per the design are represented in Table 1. The least square regression model was fitted into the responses taken from the experimental data and to define an optimization process of

Table 1
Coded levels of the independent variables used in the experimental design for microencapsulation of citronella oil.

Variables	Coded, X_i	Coded level	
		−1	1
Stirring rate (rpm)	X_1	500	750
Ratio of CO to gelatin	X_2	1:1	2:1
Amount of formaldehyde (ml)	X_3	5	15

the EE (%). The regression model equation for the EE (%) of CO that was then evaluated using the quadratic models of the form Eq. (2) was generated for each response parameter.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{j=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j-i+1}^{2} \beta_{ij} X_i X_j \quad (2)$$

where Y is the level of the measured response; β_0, β_i, β_{ii} and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction coefficients, respectively; X_i and X_j stand for coded independent variables, the main effects; X_iX_j is the interaction between the main effects.

2.7. In vitro CO release

The *in vitro* CO-releasing property was evaluated using the microcapsules produced under the maximal yield condition of microencapsulation [14]. Accordingly, a known quantity of microcapsules was placed into a known volume of water–ethanol solution (50:50). The mixture was stirred with a magnetic stirrer at a constant rate, and the temperature was maintained at 32 °C throughout the study. At a predetermined interval, i.e., at 0 min, 30 min and thereafter every hour for 10 h, 1 ml each of the receptor medium was collected, followed by the replenishment of the same volume of fresh, preheated receptor medium at each sampling interval. The collected samples were analyzed by GC/MS after extraction with ethanol. Each experiment was performed in triplicate.

2.8. Incorporation of microcapsules into ointment bases

The pure CO and the optimized microcapsules were separately formulated into ointments using four different ointment bases: white petrolatum, wool wax alcohol, hydrophilic ointment (USP) and PEG ointment (USP). All formulations with microcapsules were prepared at a concentration of 10%. All the bases, except PEG ointment (USP), were used as received and levigation technique was employed to incorporate the microcapsules. Mineral oil was used as levigating agent. PEG ointment (USP) was prepared by heating PEG 4000 (40%) and PEG 400 (60%) on a water bath to 65 °C, with continuous stirring. The mixture was allowed to cool, and the microcapsules were incorporated, with a continuous stirring until the mixture congealed.

2.9. In vitro membrane permeation

Permeation studies of the eight ointment formulations were conducted using Franz diffusion cells (Gebr.Rettberg GmbH, Göttingen, Germany). The samples were placed on a cellulose acetate membrane (0.2 μm pore size, 25 mm diameter, Sartorius, Göttingen, Germany), which was soaked in isopropyl myristate in order to mimic the lipophilic barrier, stratum corneum [20,21]. The testing sample, 300–350 mg, was placed in the donor cell, maintaining a complete and intimate contact with the membrane surface. During the study, the donor cell was covered with a slide cover glass to create an occlusive environment [22]. The receptor compartment contained a water–ethanol solution (50:50), to allow 'sink' condition and to sustain CO solubilization [23]. This compartment was constantly stirred and thermostated at 32 °C with a water jacket. The system was allowed to equilibrate for 30 min before the first sample was collected. An aliquot of receptor medium (250 μl) was collected for 6 h during the study, followed by the replenishment of same volume of fresh, preheated receptor medium at each sampling interval i.e., 0 min and 30 min and thereafter every hour for 10 h. The collected samples were analyzed by GC/MS after extraction with ethanol. Each experiment was performed in triplicate.

2.10. Statistical analysis

The results of permeation studies were treated statistically using Origin Software, Version 7. One-way analysis of variance (ANOVA) was employed for comparing the results. When there was a statistically significant difference, post hoc Tukey's honestly significant difference test was applied. A statistically significant difference was considered when $P < 0.05$.

3. Results and discussion

3.1. Gas chromatography/mass spectrometry (GC/MS) analysis

The GC/MS chromatogram of the CO is shown in Fig. 1. The major components were found to be citronellal and geraniol. GC/MS method was developed and validated using citronellal as a marker substance. The developed method was precise, accurate, specific and linear within the concentrations studied (1–40 μg ml^{-1}). Quantitative analysis of the CO indicated that the oil contains 35.3% citronellal and 25% geraniol.

3.2. Optimization of citronella oil (CO) microencapsulation by response surface methodology

Based on a three-factor-two-level full factorial design, the EE (%) of each experimental group (8 experiments, $n = 3$) was determined. The EE (%) of CO was in the range of 36.2–62.8%. The regression model equation for the EE (%) could be predicted using the following equation:

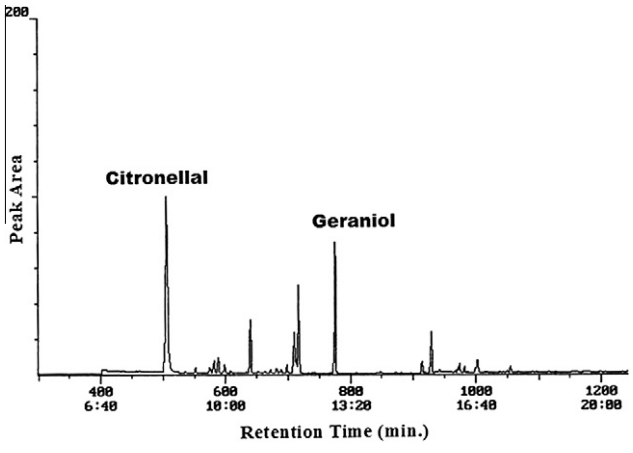

Fig. 1. GC/MS chromatogram of citronella oil. (analytical column: 30 m × 0.25 mm, coated with Optima® – FFAP – 0.25 μm film thickness; carrier gas: helium; flow rate: 1 ml min^{-1}; oven temperature: 60 °C for 1 min, increased up to 200 °C (100 °C/min) further increased to 240 °C after 14 min (30 °C/min); injector, detector, ion trap, manifold, transfer line temperature: each at 240 °C; running time: 21 min; sample solvent: chloroform).

$$\text{EE}\ (\%) = 47.7775 + 7.605X_1 + 5.22X_2 + 0.8075X_3 - 0.4075X_1X_2 - 0.3X_1X_3 - 0.205X_2X_3 + 0.3975X_1X_2X_3 \quad (3)$$

According to this statistical analysis, the stirring rate (X_1) and the oil load (X_2) in the present study were the critical factors that exerted a significant positive influence on the EE (%). In similar studies, the effects of the oil load and stirring rate on the EE (%) have been reported [14,22,24]. In the current study, the amount of formaldehyde and the various interactions of the three factors did not significantly affect the EE (%) of the CO in the range of the factors used in the experimental design. The response surface plot, Fig. 2, for the polynomial equation was constructed to find the optimum area at 5, 8, 10 and 12 ml formaldehyde. Then, three batches of model microcapsules were prepared under the optimized conditions, stirring rate of 720 rpm, CO:G of 0.57:1 and 8.15 ml of formaldehyde (37%), which provided EE (%) of 60%.

3.3. In vitro citronella oil (CO) releasing property of microcapsule

The release profile, Fig. 3, from the three batches of the model formulations were almost the same and exhibited minimum burst effect. All the microcapsules continued to release their content until the 10th hour, at which all released 70% of their content. Therefore, it could be concluded that microencapsulation can prolong the mosquito repellant action of CO for at least 10 h. The capacity of microencapsulation to prolong the persistence of volatile repellents and to change the way in which they are used for protection has been reported by other researchers [12,25].

3.4. In vitro membrane permeation

Microencapsulation decreased membrane permeation of the CO by at least 50%, Fig. 4. The phenomenon that microencapsulation retarded *in vitro* membrane permeation was consistent with the previously reported observations that showed microencapsulation retarded skin permeation of DEET [26]. According to the study [26], passive absorption and evaporation of a compound are both subjected to the same driving force, i.e., the gradient of the chemical potential of the diffusing ingredient. And, for a very thin film applied, the ratio of evaporation rate to absorption rate depends on a single dimensionless parameter χ.

$$\chi = hK_{evp}\rho/DC_{sat} \quad (4)$$

where h is the thickness of the skin, ρ is the density of the permeant, D is its diffusivity in the stratum corneum and C_{sat} is its solubility in the stratum corneum. The parameter K_{evp} is an evaporative mass transfer coefficient that depends sensitively on the vapor pressure of the permeant and the air flow over the surface. Thus, if the formulation could be structured in such a way that more surface area was exposed to the air than to the skin, then the evaporation-to-absorption rate ratio would be increased by maintaining or increasing K_{evp} and reducing the effective area for diffusion into the skin [26].

Thus, for the same reason, it can be inferred that the primary mechanism responsible for the decreased permeation of microencapsulated CO was the lower surface area achieved from microencapsulation. That is, the microcapsules that reside on the membrane surface have more surface area exposed to air than to the skin/membrane.

The observed decrease in permeation of CO from the microcapsules could also be substantiated with the fact that the percutaneous absorption of topical products involves two consecutive processes: the release from the topical preparation and absorption into and through the skin at the application site. Enhancing the release from the dosage form might promote increased percutaneous absorption. The topical bioavailability of a product therefore depends, at least in part, on the rate of release from its formulated product. Thus, as the barrier effect of microencapsulation increases the rate of release from the formulation, the percutaneous absorption of the oil from the microcapsules becomes lower than the non-encapsulated ones.

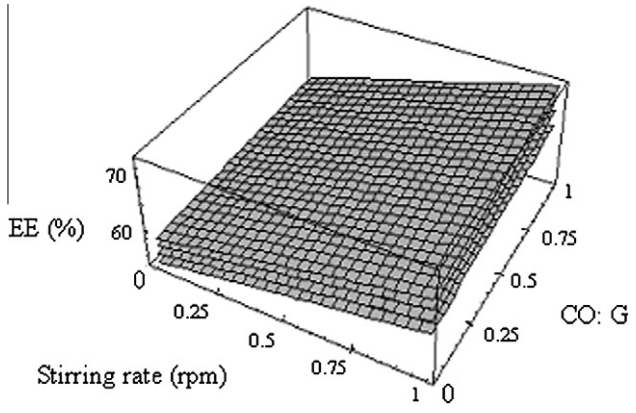

Fig. 2. Surface response of citronella oil containing microcapsules with encapsulation efficiency of 57.5%, at 5–12 ml formaldehyde (37%) level (encapsulation efficiency, EE (%); ratio of citronella oil to gelatin, CO:G).

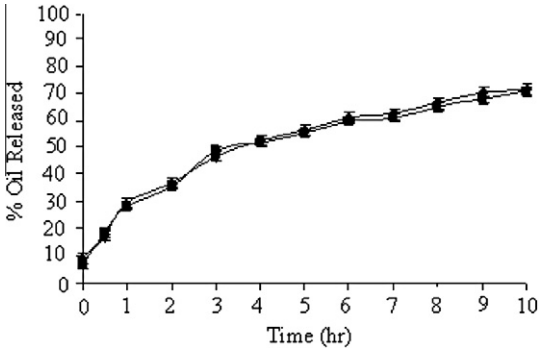

Fig. 3. Release profile of citronella oil from the optimized three batches (B1–B3) of microcapsules prepared by simple coacervation (B1, B2, B3).

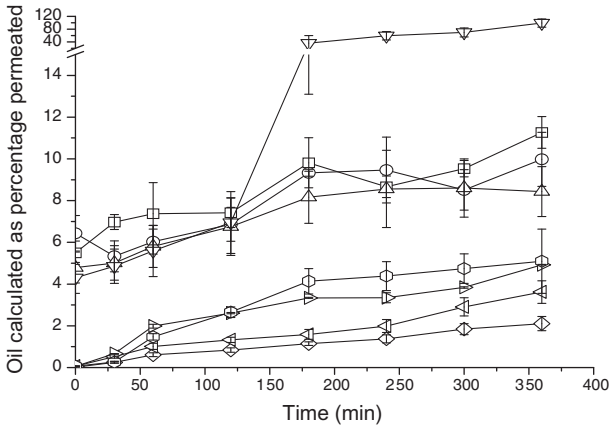

Fig. 4. *In vitro* membrane permeability profiles of non-encapsulated (NE) and microencapsulated (ME) oil dispersed in various ointment bases (White Petrolatum (NE), Woolwax Alcohol (NE), Hydrophilic Ointment (NE), PEG (NE), White Petrolatum (ME), Woolwax Alcohol (ME), Hydrophilic Ointment (ME), PEG (ME)).

The phenomena that the non-encapsulated CO showed higher permeation could best be explained by the possible and probable penetration enhancing effect of terpens. The effectiveness of terpens as permeation enhancers has been discussed elsewhere [27–31]. It was reported that geraniol, a monoterpene alcohol, provided a 16-fold increase in permeation of caffeine [28]. Furthermore, CO has been reported to contain two monoterpenoids, namely, citronellal and geraniol as its major components [32]. The present study also confirms that.

In addition, it was observed that the amount of CO permeated was dependent on the type of ointment base used. In general, as the polarity of the base increased permeation also increased, where the highest degree of release was obtained from PEG base. This could be attributed to low affinity of CO to the hydrophilic base, PEG. This, in turn, could be best explained by the partitioning of the CO between the ointment base and the receptor medium. Its affinity toward aqueous phase is reported to be much lower as compared to oil phase [33]. Thus, the CO would tend to stay in hydrophobic ointment bases like white petrolatum and wool wax alcohol. The differences in permeation from PEG and other bases in microencapsulated systems were not as high as the difference between encapsulated and non-encapsulated CO.

At the end of the study period, the membrane was extracted with chloroform to check for the amount of isopropyl myristate remained. This was done for those experiments performed with microencapsulated CO. For this purpose, a calibration curve was constructed ($Y = 13771X + 13{,}596$, $R^2 = 0.99885$, $P < 0.05$) and analysis was done by GC/MS. The amounts of isopropyl myristate recovered after completion of the experiments were calculated to be 67.04 ± 2.41, 79.61 ± 2.01, 78.28 ± 1.11 and 80.35 ± 4.12 from white petrolatum, wool wax alcohol, Hydrophilic® ointment and PEG (USP) ointment bases, respectively. Thus, the results observed during the study could not be due to the loss of the barrier effect of the isopropyl myristate.

Quantification of the remaining residues of the formaldehyde in the final ointment formulations was estimated to determine whether the residual values are below the toxic limits. Accordingly, it was calculated that a maximum of 0.15% (w/w) formaldehyde could be present. This is within the limit stated in the European Cosmetics Directive (76/768/EEC), which allows the use of formaldehyde in cosmetics up to a concentration of 0.2% (except for products for oral hygiene, where the maximum is 0.1% [34].

4. Conclusion

The results obtained showed that simple coacervation technique is a suitable method for entrapping CO. The different process and formulation variables investigated revealed that stirring rate and CO:G ratio have significant effect on EE (%). The *in vitro* release study showed that microencapsulation could control the release rate of CO. The microcapsules continued to release their content until the 10th hour, at which time all released 70% of their content. Microencapsulation decreased membrane permeation of the CO by at least 50%. It was also observed that the amount of CO permeated was dependent on the type of ointment base used. From the foregoing, it may be concluded that microencapsulation offers promising option to prolong the duration of action of CO as a potential mosquito repellent. However, this has to be confirmed through bioassays.

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