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International Journal of Pharmaceutics 253 (2003) 169–175
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Evaluation of the permeation of peat substances through human skin in vitro

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Received 18 March 2002; received in revised form 11 June 2002; accepted 16 December 2002

Abstract

Peat and various peat extracts have been successfully applied for a variety of clinical indications. Quite apart from the physico-thermal effects, new studies point towards the so-called "chemical effects" of peat containing substances. These effects include a stimulatory response of the spontaneous contractile activity (SCA) of smooth muscle (SM) tissue. The effects are, however, dependent on the possible permeability of pharmacologically active substances as naturally occurring ingredients of peat. Since peat is a mixture of various products it is necessary to examine the various peat types based upon their biological activity on SM tissue. In order to unequivocally prove the pharmacological activity of cutaneous peat treatment, in vitro permeation measurements of these actives across excised human skin can be used.

HPLC analysis revealed that aqueous peat extracts contain up to 18 fractions of water-soluble compounds of fulvic and ulmic acids. These compounds have been found to have a stimulatory response on the contractile activity of SM tissue. In vitro diffusion studies showed that the permeability of these substances across human full thickness skin (thickness: 200 um^{-1}) is highly selective and the resulting stimulatory activity is dependent on the permeated fraction. Especially, the HPLC fractions 7–11 and 14 are able to permeate human skin. Fractions 7–11 show a moderate stimulatory effect of SCA on SM for more than 90 min whereas fraction 14 shows the strongest stimulatory effect which was, however, suppressed after 87 min. These results show that the cutaneous therapy with peat treatment results in transcutaneaous permeation of biologically active fulvic and ulmic acid derivatives explaining the additional "chemical" effect of peat treatment in clinical practice.

Keywords: Peat; Smooth muscles; Permeation; Fulvic acid; Ulmic acid

1. Introduction

Peat and various peat preparations have been successfully used in the balneological practice of clinical medicine (Beer, 1998). The thermal effects (physical effects) of peat have been well examined and show clearly proven beneficial clinical effects (Kleinschmidt et al., 1985).

However, there are many indications that also a che- mical component may contribute to the clinical success of cutaneous peat treatment because several pharmacological effects have been found which cannot be contributed to the well established physico-thermal effects. Those chemical effects demonstrate antibac-

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0378-5173/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0378-5173(02)00706-8

terial properties (Naglitsch, 1983), antiviral properties (Klöcking et al., 2000), an influence on prostaglandin synthesis and stimulatory effects on spontaneous contractile activity (SCA) of smooth muscle (SM) tissue (Kauffels, 1990; Sagorchev et al., 1999; Beer and Lukanov, 1998; Beer et al., 2000a,b). Examinations of the stimulatory effects of watery peat extracts on the contractility of SM tissue have demonstrated that low molecular weight substances (<1000 Da) are responsible for the stimulatory properties. Fulmic, ulmic and humic acids, all of which have been isolated from peat, have been found to be of particular importance when considering the biological effects of peat (Beer et al., 2000a). It could be shown that these organic acid (derivatives) have an effect on the α_2 adreno and D_2 dopamine receptors (Beer et al., 2000b).

After having identified the chemical substances responsible for the found additional therapeutic effects it has now to be proven whether the permeation of these active hydrophilic substances through human skin is possible and may be the explanation for these additional effects. However, it is well known that the composition of peat in general is very complex and additionally differs depending on the source from the peat. In addition, the quality and composition of the peats is depending on many different factors as the place of origin, the primary types of the plants of origin and a whole spectrum of environmental factors. Furthermore exact structure elucidation of many compounds is hampered because they are oxidised during the extraction process and in most cases multicomponent extracts of high and low molecular compounds have been analysed. Available literature on peat compositions further indicates that gallic, vanillic and protocatechic acid derivatives are also present in aqueous peat extracts which also may be able to permeate across human skin in pharmacologically active amounts (Tuschen, 1994; Tuschen and Beer, 1995). Later studies about aqueous peat extracts show to consist predominantly of fulvic an ulmic acid derivatives which can be characterised both by HPLC and by their distinct pharmacological effects (Beer et al., 2002).

The aim of this study was to use HPLC characterised fulvic and ulmic acid derivatives obtained as watery peat extract for in vitro diffusion studies across isolated human full thickness skin and to compare the pharmacological effects of the eluates after diffusion across human skin with those of the original extract.

2. Material and methods

2.1. Sample preparation

To 100 g fresh peat (source: Wulfes Neudorf–Platendorf, Germany) 20 g bi-distilled water was added. This mixture was stored for 24 h at 25 °C. One gram of this suspension was then centrifuged (laboratory centrifuge 400R, Heraeus Instruments, Hanau, Germany) for 20 min at 3000 × *g* at 25 °C.

The upper phase was separated from the sediment and filtered through a 0.45 μM-11806-047N Filter (Sartorius, Göttingen, Germany). The resulting fraction was a clear, brownish liquid, which did not form any deposit after being left at room temperature for several weeks. Also, after being cooled to a temperature of +2 °C for several days, no deposit could be seen.

The extraction of the water-soluble components of fulvic and ulmic acids was achieved according to the method as described in literature (Beer et al., 2000a).

2.2. HPLC analysis

HPLC analysis of the water-soluble components of fulvic and ulmic acids in the native peat extract and the eluates after diffusion studies across human stratum corneum was performed as described in Section 2.1. HPLC-System of Perkin-Elmer (Arcade, NY, USA): Autosampler ISS 200; Diodenarraydetector Diode Array LC 235C; Quaterner pump Series 200 LC, Perkin-Elmer; Colonethermostat LC Oven 101 CC5904762; gas removal equipment Series 200 on liner degasser; sample collector (FC 203B; Gilson, USA); Interface 600 Link, Perkin-Elmer. Column: Analytical column Supelcosil RP C18 250 mm × 4.6 mm, 5 μm; Supelco, USA. Mobile Phases: Mobile Phase A: 99.95% acetonitrile (Merck, Darmstadt, Germany) + 0.05% trifluoroacetic acid (TFA) (Sigma Chemical Co., St. Louis, MO, USA). Mobile Phase C: 99.95% water + 0.05% TFA.

2.3. Pre-analytical preparations of samples

Five parallel samples (each 25 ml) were concentrated by evaporation at a pressure of 0.63 mbar (63 N/m^2) for 8 h at a temperature of 1 °C. Thereafter, the samples were centrifuged at 25 °C and 3000 × *g* for

a period of 10 min. The residual material was filtered through a membrane filter of a pore size of 0.45 μm and thereafter filled into 1.5 ml vials. Analysis has been done by an autosampler (ISS 200 with Interface 600 Link, Perkin-Elmer). Injected sample per analysis: 160 μl. The working parameters of the Diodarray Detectors (Diode Array LC 235C, Perkin-Elmer) were set according to the aforementioned software package Turbochrom 4.0 (PE Nelson). A two-way channelling UV-detection with corresponding wavelength was carried out (Channel A: $\lambda = 205 \pm 5$ nm; Channel B: $\lambda = 270 \pm 25$ nm). The fractions were collected in a sample collector in 25 standardised vials with a volume of 5 ml. Collection of the fractions was started directly after the sample have been injected into the HPLC-columns. The fractions-vials were automatically exchanged after each minute containing 2 ml of the mobile phase.

2.4. Measurement of the SCA of smooth muscles (SM) preparation of the muscle fibres

The specimen of SM used for the experiments were taken from the stomach of guinea pigs with a weight of approximately 350–450 g. The muscle fibres with a length of 12–14 mm and a width of 1–2 mm were taken from the corpus and antrum part of the stomach. The preparation was carried out in circular direction as far as possible according to the flow of the fibres. All animal experiments have been approved by the Ethics Commission of the Medical Faculty of the University of Plovdiv (Bulgaria).

2.5. Solutions

For the experiment, a solution of the following composition was used (mmol/l): Na^+ 143; K^+ 5.8; Ca^{2+} 3.7; Cl^- 156.2. The peat baths contained Krebs solutions of the following composition (mmol/l): Na^+ 143; K^+ 5.94; Mg^{2+} 1.19; Ca^{2+} 2.5; Cl^- 133; HCO^- 16.7; PO_4^{2-} 11.9 and glucose 11.5. Carbogenated gas with a constitution of 97% oxygen and 3% carbon dioxide was used. The pH value was 7.25. The Krebs solution in the peat baths was maintained at a temperature of 35 °C. The SCA of the SM fibres and the effects caused by the peat substances were measured under isometric conditions by means of resistance transducers according to the standardised method of Golenhofen (1976). The measured stimulation was plotted in a diagram (Fig. 3) indicating the dose–effect curves as percentage of the maximum contractile activity of the smooth muscle fibres under the influence of 10^{-5} M acetylcholine (ACH).

2.6. Preparation of human full thickness skin samples and diffusion studies

Human breast skin obtained by surgical operation was processed immediately upon arrival on the day of the surgery. After the removal of subcutaneous fat, the skin was dermatomed using an electric dermatome (Padgett Dermatome, Kansas City, USA) to a thickness of approximately 200 um^{-1}. The surface of the skin sheet was wiped clean using a tissue paper soaked with Millipore-purified water. Flow-through cells were used as described by Tanojo et al. (1997). Six cells for the diffusion experiments were equipped with dialysis membrane as supporting membrane and full thickness human skin. No influence of the dialysis membrane on the permeation of the compounds was found. The acceptor chambers of all diffusion cells were filled with 0.7 ml of the aqueous peat extract. As acceptor phase PBS solution, pH 7.4 (16 g NaCl, 2.8 g $Na_2HPO_4 \cdot 2H_2O$, 0.4 g KCl, 0.4 g KH_2PO_4, 2 g NaN_3 and 10 ml of a standard streptomycin or penicillin solution of 100 ml) was used. The flow across the acceptor phase was 4 ml/h. The temperature of the diffusion cell was kept at 32 °C.

3. Results

Fig. 1 shows a representative HPLC-chromatogram of the compounds present in the transeluates after diffusion of an aqueous peat extract (APE) across full thickness human skin in vitro (sample 7) measured at 205 nm. The result is a series of well-separated peaks which correspond to the fractions 1–18 that prove the existence of permeated substances and which are the same as those that are present in the APE before the diffusion across human skin (HPLC diagram not shown).

Fig. 2 shows typical effects of several HPLC-fractions after permeation across human skin in vitro on the SCA of SM of the corpus of the guinea-pig stomach. It can be seen that the fractions influence

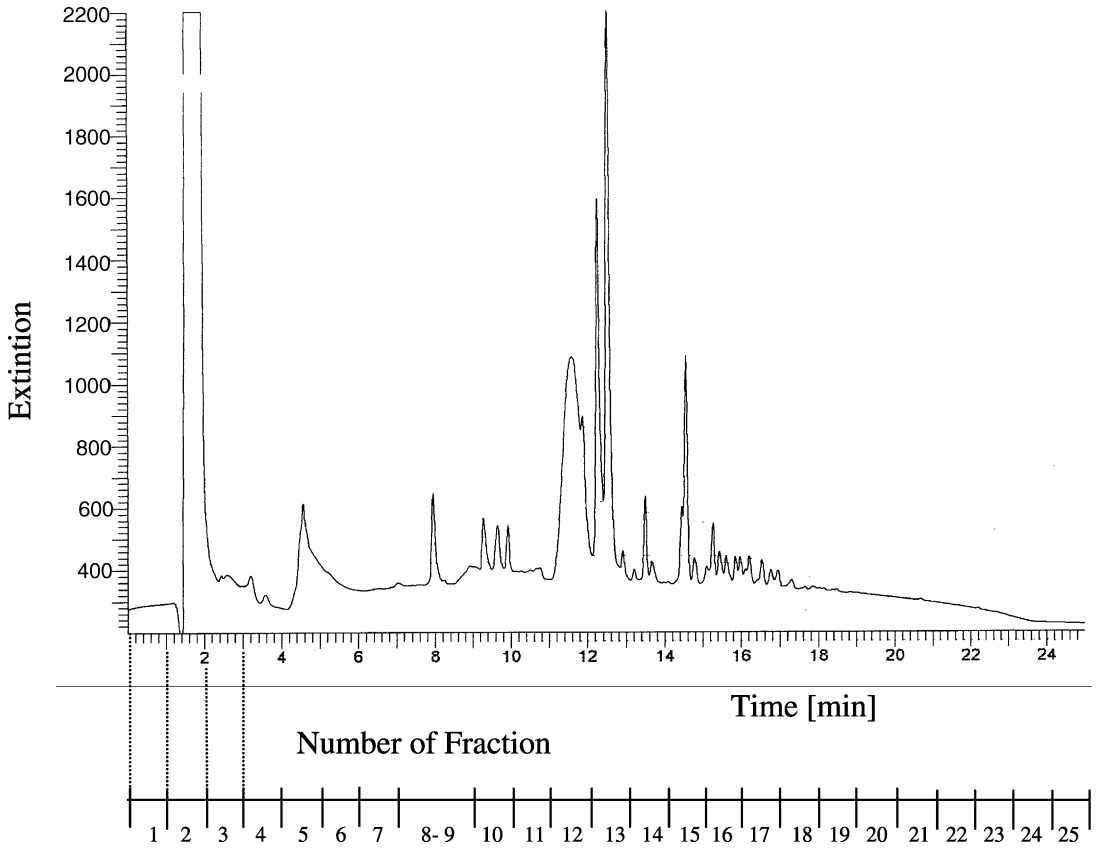

Fig. 1. HPLC-chromatogram fractions of an aqueous peat extract. Detection of the absorption of transeluates of the samples at 205 nm.

the SCA of the SM in different ways. As the diagram shows, fraction 10 has a stimulating effect on the SCA of SM, which remains unchanged for up to 87 min. Also fractions 7–9 and 11 leaves the SCA of SM unchanged even after 87 min. It can, however, be seen that after 87 min in contrast to all other fractions fraction 14 shows, that the stimulating effect is suppressed, which initially exhibited a significant stimulatory effect on the SCA of SM.

Fig. 3 shows an overview of the effects of all the fractions of both the active extracts of APE and of the transeluates on the SCA of the SM both at the beginning of the experiment (after 3 min) and after 87 min of incubation. The individual fractions have been investigated upon the individual influence of the separated fractions of fulvic and ulmic acids on the SCA of the SM. As can be seen from Fig. 3, several transeluates influence the SCA of SM of the corpus of the guinea-pig stomach. In particular, the effects of the permeated fractions 7–11 and 14 show a stimulatory effect.

From all the examined materials, it is fraction 14 that shows the greatest stimulatory effect on the SCA of SM, reaching almost 25% of the maximal contactiliy that can be registered with ACH (10^{-5} M). However, after a 87 min period, this stimulatory effect is nearly disappeared. The other fractions 7–11 leave the stimulatory effects unchanged within a time period of 90 min.

4. Discussion

The results shown in Fig. 2 demonstrate that substances found in aqueous peat extracts can permeate across human full thickness skin in quantities that have definite pharmacological effects. It could be shown that aqueous peat extracts have an stimulatory effect

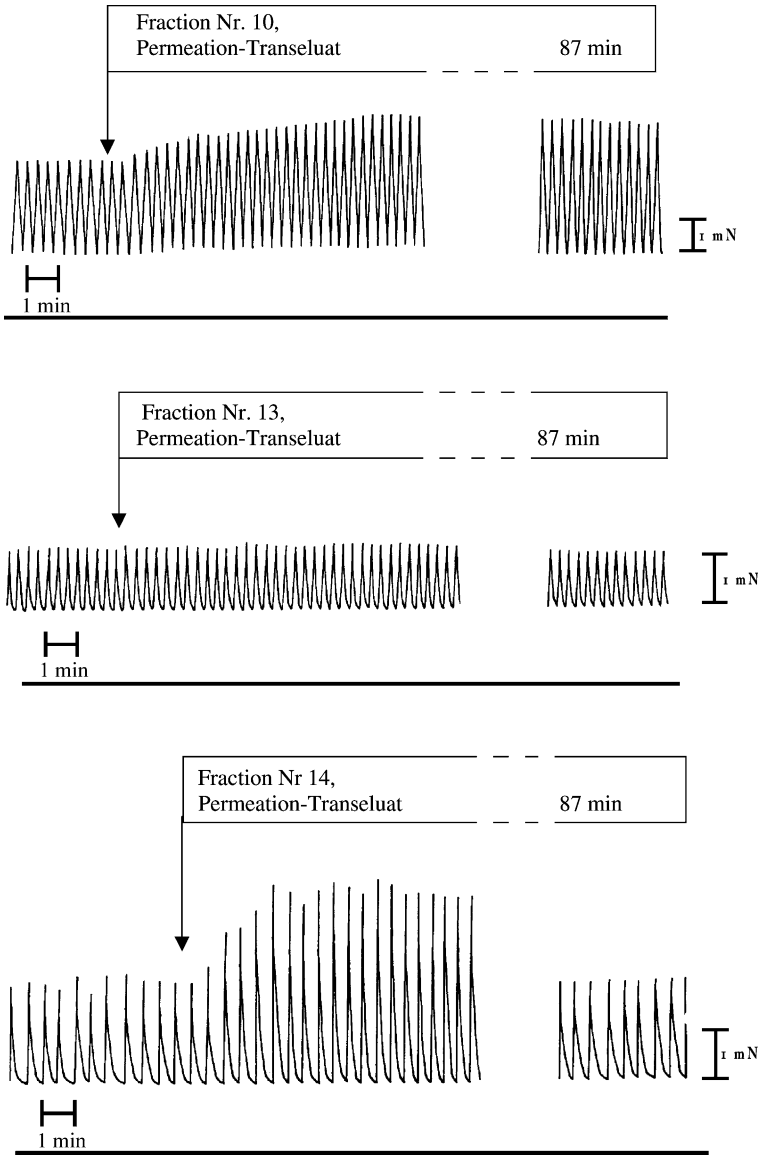

Fig. 2. Effects of HPLC fractions of the transeluates from the aqueous peat extracts on the spontaneous contractile activity (SCA) of smooth muscle (SM) tissue.

on the SCA of SM. These stimulatory effects of peat containing substances can be attributed to the activation of α_2 adrenergic receptors and D_2 dopamine receptors (Beer et al., 2000b). These biologically active substances in APE are the water-soluble components of fulvic and ulmic acids.

Fig. 3 clearly shows that human skin has a selective permeability for peat containing substances. If one compares the effects on the SCA of SM of the permeated substances with those of fractions 16 and 17 of the HPLC-fractions of ulmic acid, it can be seen that these effects cannot be found in the corresponding permeated fractions. The same statement can be made with regard to fulvic acid in the HPLC fractions 12, 13 and 15. The corresponding permeated fractions have negligible effects on the SCA of SM.

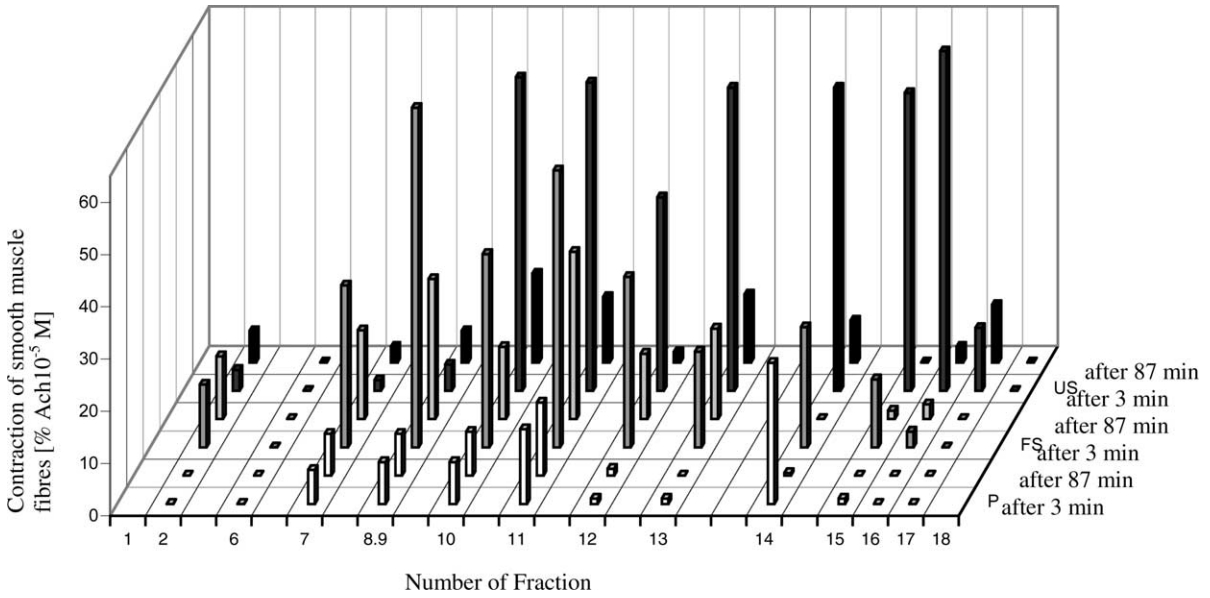

Fig. 3. Effects of the HPLC-fractions of the permeations-eluates (P) of fulvic (FS) and ulmic acid (US) on the spontaneous contractile activity (SCA) of smooth muscle (SM) fibres both at the beginning of the experiment (3 min, 1st column) and after a 87 min time span (2nd column).

Pharmacologically active HPLC fractions can be divided into two groups according to their method of action: The first group includes the fractions 7–11 which demonstrated a stimulatory response of the SCA of SM that remained unchanged after 90 min, whereas the corresponding fulvic and ulmic acid fractions show a reduction in the stimulatory effects during this time span.

Fraction 14 is the only fraction that can be said to belong to a second group because of its different pharmacological action. From all other fractions it is only fraction 14 that shows a very strong stimulatory effect on the SCA of SM, being almost 25% of the maximum contractility that can be measured with ACH (10^{-5} M) alone. However, this stimulatory effect becomes negligible after 90 min. This pharmacological response pattern is similar to that of fraction 14 of the aqueous peat extract containing fulvic acid. For this compound it already could be shown to have partial agonist effects on α_2 adrenergic receptors and D_2 dopamine receptors (Beer et al., 2000a).

These phenomena can be attributed to the special properties of the α_2 adrenoreceptors. Binding of agonists to receptors leads to conformational changes of the receptors. This provokes a decline in the levels of cyclic AMP which in turn leads to the contraction of smooth muscle cells (Zhang et al., 1990). The conformation change is also recognised by a kinase which causes the phosphorylation of the polypeptide chain of the receptors. The agonist is then unable to bind onto the receptor (Robert, 1998; Ulrik and Brian, 1998). In this form, the complex is able to travel via endocytosis through the cell membrane into the cell (Robert, 1998).

A constant influence of adrenergic agonists on the effector cell leads to changes with regard to the receptor density and affinity, which is linked to reduced physiological reactions of the receptor, an effect also referred to as “tachyphylaxia” or “hyposensitising” (Lansberg and Young, 1998).

The permeatied fractions across isolated human skin are very likely (as can be expected) to possess significantly lower concentrations of biologically active substances as compared to the concentrations of the water soluble components of fulvic and ulmic acids in the unpermeated aqueous fraction (Fig. 3). This can be confirmed when the stimulatory effects on the SCA of SM are compared with those of fulvic and ulmic acid fractions. Here a three- to six-fold difference in activity can be observed. Using significantly lower

concentrations of agonists, the described self-inhibitory effect of the receptor is not able to take place, at least not in the time interval of 90 min which was used for these experiments. In this respect the stimulatory effect of the fractions on the SCA of SM remain unchanged.

5. Conclusion

In conclusion the following three statements can be made: Firstly, it could be shown that a transdermal passive permeation of water soluble peat substances is obvious to take place and that as a consequence of this pharmacologically active substances are able to enter into the organism in quantities that are sufficient to cause biological effects. Secondly, it is evident that human skin possesses a selective permeability for the water-soluble fulmic und ulmic acids and derivatives fraction isolated as peat extract. And thirdly a good explanation has been found why the cutaneous application of peat plays an important role with respect to beneficial therapeutic effects when used in clinical practice.

In this paper we aim to show that a permeation of active peat agents across human skin is a fact. For the future we plan to further investigate, especially fraction 14 in more detail, by HPLC because it seems to be the most important fraction responsible for the pharmacological action. The resulting substances (especially fractions 6–8) will then be identified with mass spectrometry. This will allow further discussion as to the active peat agents and to the permeation modulation as shown by human skin.

Acknowledgements

The technical assistance of Mr. P.E.H. Roemele for skillfully performing the in vitro diffusion studies is very much acknowledged.

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