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An evaluation of antifungal properties of peppermint water

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Summary

The use of peppermint water as a flavouring agent in extemporaneously prepared alkaline mixtures has been implicated with bacterial contamination and proliferation in these mixtures. The natural tendency therefore, has been to disfavour the use of peppermint water as a flavouring agent. The British Pharmacopoeia (1973) recommends a 1:20 dilution (5.0%) of concentrated peppermint water (CPW) for use in mixtures. This concentration has now been shown to exhibit considerable fungistatic but not fungicidal activity against strains of *Aspergillus niger* and *Penicillium chrysogenum*. Fungicidal concentrations of CPW as obtained in the present study are, however, well in excess of the concentrations normally included in mixtures and they completely halted spore germination at any given phase of growth without affecting size of swollen spores.

Introduction

Peppermint water is used as a flavouring agent in BP alkaline mixtures and as a carminative in many pharmaceutical preparations. The Public Health Laboratory Service Working Party, in a survey of bacterial contamination of samples of medicines intended for oral and topical use by hospital patients, reported that *Pseudomonas aeruginosa* was found in 2.7% of samples and constituted the greatest potential hazard. It was found most often in peppermint water and in alkaline mixtures flavoured with peppermint. Others, Beveridge and Hope (1971), Yasmin et al. (1971) and Hugbo (1977), have also reported the supportive effect of peppermint water on bacterial growth. Hugbo (1977) observed that peppermint water, however, exhibited considerable inhibitory action against unidentified strains of *Penicilium*

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and *aspergillus* moulds isolated from the local environment during the course of an investigation into the growth of micro-organisms in a magnesium trisilicate mixture. The present report presents further data in support of these observations.

Materials and methods

Two mould species were used; these were *Aspergillus niger* and *Penicillium chrysogenum* both of which were isolated from contaminated liquid medicines. They were grown on Sabouraud Dextrose Agar (SDA) Slants at 30°C. Spores were harvested after 5 days, washed by centrifugation, dispersed with sterile glass beads and stored at 4°C after standardization by haemocytometer counts. Harvesting of the spores was affected by carefully scraping off spores in the presence of 5 ml water, by means of a sterile, L-shaped platinum wire such that minimal mycelia were carried along into the supernatant as evidenced by microscopic examination during the counting procedure.

Concentrated peppermint water (CPW) (B.P., 1973) was prepared from peppermint oil (Evans, Speke) and sterilized by autoclaving in tightly sealed bottles, capped with aluminium tops.

Three parameters were selected for investigation: (a) suppression of mycelial growth in solid medium; (b) inhibition of growth of spores in liquid medium; and (c) inhibition of spore swelling in liquid medium.

Suppression of mycelial growth on solid medium

Graded volumes, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml (representing graded concentrations) of CPW were incorporated into molten SDA to give a total volume of 20 ml, poured and allowed to set in petri plates. Each plate was flooded with spore suspension, 1.0×10^5 spores $\cdot$ ml^{-1} and the excess suspension carefully pipetted off. The plates were incubated at 30°C for 5 days.

In order to reduce loss of volatile constituents from the peppermint water over the period of incubation, 10 ml agar overlay containing identical concentrations CPW as were used in each determination was applied to each plate after the agar had set. Preliminary determination of the effect of the agar overlay without CPW had shown that it neither affected growth of the spores nor formation of *Mycelia* to any significant extent.

Determination of fungistatic and fungicidal concentrations of peppermint water

Sabouraud liquid medium (SLM) and chemically defined medium (ChDM) were used. The latter which consisted (g/l) of the following: glucose, 10; $(NH_4)_2HPO_4$, 0.2; NaCl, 5.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.4, adjusted to pH 7.4 with 1 M NaOH was found to support prolific growth of both moulds. A series of tubes containing graded dilutions of CPW in each tube of medium were inoculated with 1×10^5 spores $\cdot$ ml^{-1}, final volume 10 ml and incubated at 30°C for 5 days. All tubes showing no growth were re-subcultured by transfer of 1.0 ml into 9.0 ml appropriate medium and incubated for an additional 5 days. The highest dilution of

subcultured tubes showing no growth were regarded as containing fungicidal concentrations of CPW. Similarly the highest dilution of the first set of tubes showing no growth were regarded as fungistatic concentration.

Determination of inhibition of spore swelling

Inhibition of spore swelling was determined by measuring changes in spore sizes prior to and during germination of the spores of *Aspergillus niger* only. The microscopic method of particle size measurement using a calibrated eye piece and graticule was employed. The suitability of the sizing procedure (S.D. = 0.08, coefficient of variation CV = 2.09%) was first assessed by sizing 20 washed, ungerminated spores suspended in 1/4 strength Ringer's solution. The effect of different concentrations of CPW on *Aspergillus niger* spores was then assessed as follows.

Different volumes of CPW were added to 100 ml capacity conical flasks containing predetermined amounts of SLM to give 9.5 ml in each flask. The flasks were inoculated with 0.5 ml spore suspension, and incubated at 37°C (McRobbie and Parker, 1974) using a slow agitation of 25 throws $\cdot$ min^{-1} on a reciprocal water bath. Samples were taken at hourly intervals for up to 8 h and sizes of the spores were measured. Effect of CPW on already swollen spores was also measured by inoculation of 0.5 ml spore suspension into 7.0 ml SLM and incubating the mixture at 37°C for 4 h. Thereafter, 2.5 ml CPW was added and the reaction mixture allowed to incubate for a further 12 h. Spores were sized at 1, 2, 4, 8 and 12 h after the addition of CPW.

Results

The test for the ability of peppermint water to suppress *Mycelial* production proved positive for the two species of moulds used. There was increasing inhibition of mycelial formation as the concentration of peppermint was raised from 0.0 to 2.5, 5.0, 7.5 and 10.0% v/v. This was clearly shown by the scantiness of the growth which, however, was more pronounced for *Penicillium chrysogenum* than it was for *Aspergillus niger*, indicating that the former is more sensitive to peppermint water.

Conversely, the control plates which contained no peppermint water produced profuse growth of mycelia and sporulations at the 5-day incubation at 30°C. When the peppermint water concentration was 10%, production of mycelia was almost completely abolished for *Aspergillus niger* and totally abolished for *Penicillium chrysogenum*. No spores were present at all at this concentration.

Table 1 shows the fungistatic and fungicidal concentrations of CPW in SLM and in ChDM. There appears to be no marked effect of the constituents of the liquid medium on the antifungal activity of CPW.

Thus the fungistatic concentration in SLM and ChDM are 4.0 and 3.5%, respectively, for *Aspergillus niger* and 2.5 and 3.0% for *Penicillium chrysogenum*. Fungicidal activity against *Aspergillus niger* was displayed by concentration of 22.5% CPW in SLM and 16.5% in ChDM while the values for *Penicillium chrysogenum* were 12.5% and 14.5% CPW. The ratio of fungicidal to fungistatic concentration for

TABLE 1

FUNGISTATIC AND FUNGICIDAL CONCENTRATIONS OF CONCENTRATED PEPPERMINT WATER AGAINST MOULDS IN LIQUID MEDIA

Organism	Concentrations of CPW (% v/v) producing observed activity					
	Sabouraud liquid medium (SLM)			Chemically defined medium (ChDM)		
	Fungi-static	Fungi-cidal	Ratio cidal-stati	Fungi-static	Fungi-cidal	Ratio cidal-static
Aspergillus niger	4.0	22.5	5.6	3.5	16.5	4.7
Penicillium chrysogenum	2.5	12.5	5.0	3.0	14.5	4.8

each organism in different media is calculated and also given in Table 1. It can be seen, that these ratios are approximately identical in magnitude particularly in relation to a given medium.

The effect of CPW on depression of spore size of *Aspergillus niger*, before and during germination is shown on Tables 2 and 3. There was complete inhibition of spore swelling when the concentration of CPW was 25% (v/v) which is about the same level as the fungicidal concentration for this organism. With weaker concentrations the rate of swelling was depressed relative to the concentration of CPW (Table 2). A control determination showed there was always a lag period of 60–90 min before the onset of germination. Spores which were allowed to swell for 4 h (8.0 μm) before the addition of CPW (25.0%) were completely halted in that phase when the CPW was added (Table 3). There was no variation in sizes of the swollen spores even after 12 h of re-incubation.

TABLE 2

EFFECT OF PEPPERMINT WATER ON SPORE SWELLING OF *Aspergillus niger* AT 30°C IN SABOURAUD LIQUID MEDIUM

Conc. peppermint water (% v/v)	Mean spore sizes (μm) at:					
	0 h	1.0 h	2.0 h	3.0 h	4.0 h	8.0 h
0.0 (control)	4.0	4.02	5.0	6.1	8.0	10.2
5.0	3.75	4.1	4.8	5.0	6.3	7.5
10.0	4.10	4.0	4.2	5.0	6.1	6.0
15.0	4.0	4.0	4.2	4.3	4.3	5.5
20.0	4.0	4.0	4.1	4.25	4.3	5.0
25.0	4.1	4.0	4.0	4.1	4.1	4.0

TABLE 3

INVARIANCE IN THE SPORE SIZE OF *Aspergillus niger* IN THE PRESENCE OF A FUNGICIDAL CONCENTRATION (25%) OF CPW ADDED AFTER PREINCUBATION FOR 4 h

Time (h) of incubation after addition of CPW	Spore size (μm)	
	Control	Test
0	8.0	8.1
1	8.3	8.0
2	9.0	8.02
4	9.8	8.3
8	11.4	8.0
12	-	8.0

Discussion

For a proper evaluation of an antifungal agent, it is necessary to maximize all favourable conditions before proceeding with the tests. Such conditions include an adequate incubational period, temperature and suitable culture medium. These will enable the antifungal effect of the agent to be studied under the full growth of the test organism.

Preliminary studies on this report showed that there was hardly any difference between SLM and ChDM in relation to repressive action of CPW on growth of the moulds employed. Growth at 30°C for 5 days in the absence of CPW on solid medium (SDA) produced prolific sporulation and mycelia. However, there was a definite difference between concentrations of the drug acting in solid medium (SDA) and that acting in liquid medium (SLM) as can be seen from the fact that 10% CPW was required to produce fungistatic effect on *Aspergillus niger* in agar dilution while 5.0% produced the same effect in the broth dilution method.

The minimum fungistatic and fungicidal concentrations clearly reveal that *Penicillium chrysogenum* is more sensitive to CPW than *Aspergillus niger*. When the ratio of fungicidal to fungistatic concentrations are calculated for each organism, they are found to be approximately equal, which suggests a possible equimolecular concentration for lethal doses of CPW at the sites of action within the spore. The large difference between the fungistatic and fungicidal concentration therefore probably reflects a permeability barrier offered by structures external to the underlying membrane.

Inhibition of growth as measured by absence of turbidity merely represents a gross effect on antifungal activity. Spore swelling is the first during a germination process leading to vegetative forms. An antifungal agent could act by inhibiting spore germination or by inhibiting growth of the vegetative forms after spore germination. This antifungal effect could be at the spore level or at the vegetative level. A more sensitive index therefore used in the evaluation of antifungal activity is the depression of spore swelling during early development.

Present results (Table 2) show that there was consistently a depression of spore swelling, the rate of which increased with increase in concentration of CPW. A concentration of 20%, i.e. 2.0 ml in 10 ml SLM, exhibited the greatest depression while 25% (2.5 ml in 10 ml SDA) completely abolished spore swelling even after 12 h. This concentration has previously been found to be the minimal fungicidal concentration; the spore may be presumed dead at this concentration of CPW. Spores, which had already swelled before CPW (25% concentration) was added, were completely halted in that phase; there was neither increase nor decrease in size. It therefore seems that the fungicidal action of peppermint water could be brought about by a mechanism that prevents germination without involving 'shrinkage of the spores'.

The results show that peppermint water exhibits considerable antifungal activity which could prove useful in alkaline mixtures which are prone to contamination by fungi, expecially those types which are found in humid tropical climates.

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