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Antimicrobial action of essential oils : the effect of dimethylsulphoxide on the activity of cinnamon oil

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P. HILI, C.S. EVANS AND R.G. VENESS. 1997. Fifty-one essential oils extracted from plants of known origin were tested for their antimicrobial activity against three bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and four yeasts, *Torulopsis utilis*, *Schizosaccharomyces pombe*, *Candida albicans* and *Saccharomyces cerevisiae* using the drop diffusion method. All showed antimicrobial activity against at least one of the micro-organisms. Following this preliminary screening, 13 essential oils showing antimicrobial activity against at least five of the micro-organisms were tested in the range 50 μg ml^{-1} to 500 μg ml^{-1} using broth micro dilution techniques with dimethylsulphoxide (DMSO) as a dispersing solvent. The concentration of most of the oils required for total inhibition of growth was >500 μg ml^{-1}. Further studies on the antimicrobial action of cinnamon oil in the range 10–150 μg ml^{-1} showed that 50-fold higher activity was found when no dispersing solvent was used.

INTRODUCTION

Essential oils are the odorous, volatile products of plant secondary metabolism, normally formed in special cells or groups of cells or as glandular hairs, found on many leaves and stems. Oils occur as a globule or globules in the cell, and may also be excreted from cells lining the schizogenous ducts or canals. They may be present in such glandular cells or ducts in any or all organs of the plant including roots, stem, buds, leaves, flowers and fruits. However, oils are commonly concentrated in one particular region such as leaves, bark or fruit, and when occurring in various organs in one plant may possess different individual chemical components (Bonner 1991).

The function of essential oils is believed to be largely communicative and a variety of complex interactions have evolved enabling plants to utilize essential oils to influence their environment. For example, the volatile monoterpenes that comprise about half of the oleoresin produced by certain species of conifer act as phytoprotective agents, defending the tree from herbivore and pathogen attack (Gijzen *et al.* 1991). Essential oils also act as antifeedants, with components of oils of shrubs and conifers inhibiting rumen micro-organisms upon which ruminants rely for digestion of plant material (van Beek 1986).

The use of essential oils as antimicrobial agents has been described qualitatively for many years starting with the phenol coefficients of a number of essential oils published by Martindale (1910). Recently, it has been demonstrated that essential oils of female and hermaphrodite *Thymus bacticus* Boiss (thyme oil) showed marked activity against some Gram-positive and Gram-negative bacteria and yeast. The activity was greater in the essential oils containing larger amounts of geraniol (Cruz *et al.* 1993). The antifungal activity of six essential oils was tested *in vitro* and 16 clinical isolates of the dermatophytes *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* isolated from patients with dermatophytosis (Lima *et al.* 1992). The oils were obtained from the plants *Annona classifora*, *Cinnamomum zeylanicum*, *Cymbopogon citratus*, *Ocimum gratissimum*, *Protium heptaphyllum* and *Xylopin frutesens*. Oils (with the exception of that from *P. heptaphyllum*) caused inhibition of 81% of the studied strains of dermatophytes. Only two strains of *T. rubrum* and one of *M. canis* were resistant.

The antimicrobial properties of oils from *Melaleuca alternifolia* (tea tree), *Mentha piperita* (peppermint) and *Salvia officinalis* (sage) were the most potent against facultatively anaerobic oral bacteria. Thymol and eugenol were the potent essential oil components (Shapiro *et al.* 1994). Within the

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literature there is some discordance between the level of antimicrobial activity reported for various essential oils. Data such as the geographical origin and the exact composition of the essential oils studied is rarely cited so that no consideration can be given to the basic question of what are the principles responsible for therapeutic action (Janssen *et al.* 1987). The importance of geographical conditions has been recently demonstrated in studies of two lemongrass species, *Cymbopogon nardus* (L.) and *Cymbopogon pendulus* grown under mild and moderate water stress for 45 and 90 d, that showed significant differences in the levels of the major oil constituents, geraniol and citral. The composition of the oils varied depending upon the level and duration of moisture stress (Singh-Sangwan *et al.* 1994).

Comparison of methodologies has shown that agar diffusion and serial dilution methods to measure antimicrobial activity do not always give parallel results, confusing any assessment of antimicrobial activity (Youssef and Tawil 1990). Reproducibility is particularly difficult using the drop diffusion method (Carson and Riley 1995). Factors such as solubility and rate of vaporization of the oils are difficult to monitor and may lead to erroneous results.

The aim of this study was to examine the antimicrobial activity of 51 essential oils of known geographical origin, against a range of Gram-positive and Gram-negative bacteria and yeasts. The effect of the addition of dimethylsulphoxide (DMSO) as an oil solubilizer on the antimicrobial activities was investigated.

Furthermore, by determining the composition of each oil by gas chromatography the chemical components associated with the antimicrobial action could be identified.

MATERIALS AND METHODS

Essential oils

Fifty-one essential oils from a variety of commercial sources were tested. The species of plant, geographic origin and the part of the plant from which the oil was obtained are shown in Table 1.

Gas chromatography

To 400 μl of each essential oil was added 25 μl of isopentane and 25 μl of eicosane to act as low and high end markers respectively. The analyses were performed using a Perkin Elmer 8600 gas chromatograph fitted with flame ionization detector. A 50 m column 0·22 m i.d. with 1 μm of BP1 phase (SGE) was used; oven temperature was 70° to 260°C at 2°C min^{-1}; detector and injector temperatures were 300°C; amount injected was 0·1 μl (split ratio 15 : 1); carrier gas was helium at a flow rate of 1·738 ml min^{-1}; total run time was 95 min.

Media

Oxoid nutrient broth (NB) and nutrient agar (NA) used for bacterial cultures were made up according to the manufacturer's directions. Malt extract (Oxoid), 3 g, yeast extract (Oxoid), 3 g, glucose (BDH), 10 g, and mycologicol peptone (Oxoid), 5 g, were made up in 1 l of distilled water and autoclaved at 121 psi for 15 min (MYGP broth). MYGP agar was made up as the broth with the addition of 20 g l^{-1} of Oxoid Agar No. 3.

Micro-organisms

The micro-organisms used in this study were *Staphylococcus aureus*, NCTC 6571, *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* NCTC 10662, *Schizosaccharomyces pombe* CMI 124, *Saccharomyces cerevisiae* CMI 61302, *Candida albicans* NCPF 113 and *Torulopsis utilis* CMI 23311. The bacteria were maintained at 4°C on slope cultures (NA) and the yeasts were maintained at 4°C on MYGP agar.

Antimicrobial activity study

To establish growth curves, organisms were transferred aseptically from stock slopes into 9 ml of broth and incubated at 30°C before dilution to 10^6 organisms ml^{-1}. Spread plates were prepared using 200 μl of broth. A 5 μl drop of essential oil was placed in the centre of each plate and the plates incubated at 30°C for 24 h, after which the diameter of inhibition of growth in mm was measured. A minimum of three replicate plates was used for each oil.

Broth micro dilution assay

The essential oils were prepared in DMSO. A stock solution of the oil to be tested was prepared by diluting 0·336 g in 20 ml of DMSO. Stock solution was then added to the culture broth to give final concentrations of 10–500 μg ml^{-1}. To 2·0 ml of nutrient broth was added the required amount of oil in DMSO and 200 μl of the test bacteria diluted to 10^5 organisms ml^{-1}. The samples were then incubated for 40 h at 37°C and the absorbances read at 700 nm. Control samples of broth and organism, and broth with DMSO plus organism (DMSO blanks) were incubated under the same conditions.

To 2 ml of MYGP broth was added the required amount of oil in DMSO and 200 μl of the yeast in MYGP broth. The samples were incubated for 40 h at 30°C and the absorbances read at 700 nm. Control samples were incubated under the same conditions. The minimal inhibitory concentration (MIC) was defined as the lowest concentration which resulted in a reduction of $>90\%$ in the observed absorbance.

Table 1 Botanical and geographical sources of essential oils

Oils	Species	Part of plant	Country of origin	Extraction method
Aniseed	*Illicium verum*	Fruit	China	Steam distillation
Basil	*Ocimum basilicum*	Herb	India	Steam distillation
Bergamot	*Citrus bergamia*	Rind	Ivory Coast	Pressed
Black pepper	*Piper nigrum*	Fruit	India	Steam distillation
Cajuput	*Melaleuca cajiputi*	Twig/leaf	Vietnam	Steam distillation
Cardamon	*Elettaria cardamomum*	Fruit	Guatamala	Steam distillation
Cedarwood	*Cedrus atlantica*	Wood	France	Steam distillation
Chamomile (blue)	*Matricaria chamomilla*	Flowers	Egypt	Steam distillation
Chamomile roman	*Anthemis nobilis*	Flowers	Hungary	Steam distillation
Cinnamon	*Cinnamomum zeylanicum*	Leaf	Sri Lanka	Steam distillation
Clary sage	*Salvia sclarea*	Herb	France	Steam distillation
Clove	*Eugenia caryophyllata*	Leaf	Madagascar	Steam distillation
Coriander	*Couiandrum sativum*	Fruit	Russia	Steam distillation
Cypress	*Cupressus sempervirens*	Needles	France	Steam distillation
Eucalyptus	*Eucalyptus globulus*	Leaf	France	Steam distillation
Eucalyptus citriadora	*Eucalyptus citriodora*	Leaf	China	Steam distillation
Fennel	*Feniculum dulce*	Seed	Spain	Steam distillation
Geranium	*Pelargonium graveolens*	Herb	Egypt	Steam distillation
Ginger	*Zinger officinalis*	Root	China	Steam distillation
Grapefruit	*Citrus paradisi*	Rind	USA	Pressed
Ho leaf	*Cinnamonum comphora*	Leaf	China	Steam distillation
Jasmine	*Jasminun officinalis*	Flowers	Morocco	Solvent extraction
Juniper	*Juniperus communis*	Twigs/berries	France	Steam distillation
Juniperberry	*Juniperus communis*	Berries	France	Steam distillation
Lavender	*Lavendula hybrida*	Flowers	France	Steam distillation
Lemon	*Citrus limonum*	Rind	Italy	Pressed
Lemongrass	*Cymbopogon citratus*	Herb	Nepal	Steam distillation
Lime	*Citrus aurantfolia*	Rind	West Indies	Pressed
Mandarin	*Citrus reticulata*	Rind	Brazil	Pressed
Marjoram	*Thymus masticina*	Herb	Spain	Steam distillation
Melissa	*Melissa officinalis*	Herb	France	Steam distillation
Myrhh	*Commiphora myrrha*	Resin	Ethiopia	Steam distillation
Neroli	*Citrus aurantium*	Flowers	Morocco	Steam distillation
Olibanum	*Boswellia thurifera*	Resin	Somalia	Steam distillation
Orange	*Citrus sinensis*	Rind	Corsica	Steam distillation
Palmarosa	*Cymbopogon martini*	Grass	Indonesia	Steam distillation
Patchouli	*Pogostemon patchouli*	Leaves	USA	Steam distillation
Peppermint	*Mentha piperita*	Herb	India	Steam distillation
Petigrain	*Citrus aurantium*	Twigs/leaves	S. America	Steam distillation
Pine	*Pinus sylvestris*	Needles	France	Steam distillation
Rose	*Rosa damascena*	Petals	Morocco	Steam distillation
Rose absolute	*Rosa damascena*	Petals	Morocco	Steam distillation
Rosemary	*Rosmarinus officinalis*	Herb	France	Steam distillation
Sage	*Salvia officinalis*	Herb	Spain	Steam distillation
Sandalwood	*Santalum album*	Wood	India	Steam distillation
Tagettes	*Tagettes glandulifera*	Herb	Nepal	Steam distillation
Tea tree	*Melaleuca tenifolia*	Leaves	Australia	Steam distillation
Thyme red	*Thymus vulgaris*	Herb	France	Steam distillation
Vetiver	*Vetuvera zizanoides*	Grass	China	Steam distillation
Ylang ylang	*Canaga odorata*	Flowers	Commores	Steam distillation

Assay of cinnamon oil with and without DMSO as solubilizer against *Saccharomyces cerevisiae*

To 50 ml of MYGP broth in 250 ml conical flasks was added the required amount of cinnamon oil in DMSO (prepared as in the broth micro dilution assay). Parallel samples added the oil without DMSO to 50 ml MYGP broth. To each sample 5·0 ml of *S. cerevisiae* diluted to give 10^5 organisms ml^{-1} in the final solution were added. The samples were incubated upright for 48 h at 30°C on a rotating platform operating at 100 rev min^{-1}. Following incubation, absorbance was measured at 700 nm and the mass of cells determined by centrifugation followed by freeze drying. Control samples of broth, broth + organism, and broth + oil were incubated under the same conditions.

Analysis of data

For the drop diffusion method a minimum of three replicates was prepared. The mean and standard error were calculated. For the broth dilution method the mean absorbance of the triplicate samples was compared with the mean absorbance of the broth samples containing DMSO without oil to give a measure of the overall reduction in growth. For the comparative assay with and without DMSO, analysis of variation (ANOVA) was performed followed by Student *t*-tests.

RESULTS AND DISCUSSION

Of the 51 oils tested all showed activity in inhibiting the growth of at least one of the seven micro-organisms by the drop diffusion method. Thirteen oils demonstrating activity against at least five of the organisms are shown in Table 2.

The oils inhibited growth of the yeast species more than the bacterial species confirming previous work (Hammerschmidt *et al.* 1993). *Pseudomonas aeruginosa* proved to be the most resistant organism with only 13 of the 51 oils showing any inhibitory activity, which correlated with previous data (Panizzi 1993). *Staphylococcus aureus* proved to be the second most resistant organism, whereas 20 oils showed activity against *E. coli*. In drop diffusion experiments studying the inhibition of growth of the yeasts, 47 oils showed inhibitory activity against *C. albicans*, 49 oils showed activity against *S. pombe* and 50 showed activity against *S. cerevisiae* and *T. utilis*. The level of inhibitory activity of the oils varied from 0·3 to 90% of total growth.

Analysis of each oil by gas chromatography showed that similar chemical constituents were found in some of these oils. A single chemical species, however, was not ubiquitously found suggesting that different constituents were contributing to the antimicrobial activity of each of the oils.

The drop diffusion method proved useful as a preliminary screening test for such a large number of oils. However, because of its reliance on the solubility and the rate of diffusion of an oil in aqueous medium, low zones of inhibition could often falsely arise (Janssen *et al.* 1986). Further tests using broth dilution assays were made to ensure that the solubilizing agent itself did not have an inhibitory effect, or if so, that this effect was minimized. The concentration of DMSO used in the broth dilution assays was kept below 30 $\mu l\ ml^{-1}$, selected from a preliminary screening of the level of DMSO to ensure the effects on bacterial and yeast growth were minimal.

The results of the broth dilution assays are shown in Table 3. Above 90% reduction in growth was seen with clove oil

Table 2 Antimicrobial effect of essential oils using the drop diffusion method

Oil	*E. coli*	*Staph. aureus*	*Ps. aeruginosa*	*C. albicans*	*S. cerevisiae*	*T. utilis*	*S. pombe*
Clove	37·8	21·5	22·8	40·3	49·7	39·0	34·3
Coriander	13·2	11·7	6·0	28·7	32·0	37·0	33·0
Cinnamon	44·9	45·2	25·4	38·7	53·0	42·3	43·0
Cardamon	5·0	2·7	0	7·3	31·7	18·7	*
Thyme	40·6	51·3	14·5	60·7	80·0	67·3	69·0
Tea tree	13·7	6·9	5·3	11·3	12·7	47·0	20·3
Marjoram	21·0	12·0	3·0	9·0	22·7	7·7	22·7
Ho leaf	16·0	15·5	4·7	37·3	40·0	34·0	47·7
Rosemary	1·3	0	0	6·7	12·3	9·7	16·3
Peppermint	16·1	16·0	4·8	16·0	13·0	13·3	17·0
Palmarosa	0	13·7	7·0	29·7	21·0	23·3	23·0
Lemongrass	*	*	*	*	*	47·0	*
Sage	15·0	5·3	0	11·0	9·0	12·7	15·7

Mean diameter of inhibition of growth in mm, standard error within 10%. Maximum plate diameter was 80 mm.
*Denotes patchy inhibition but no single clear zone.

Table 3 % Reduction in growth caused by essential oils at 500 μg ml^{-1} in broth dilution tests for antimicrobial activity

Oil	*E. coli*	*Staph. aureus*	*Ps. aeruginosa*	*C. albicans*	*S. cerevisiae*	*T. utilis*	*S. pombe*
Clove	74·3	82·9	74·5	60·9	99·0	94·6	93·8
Coriander	67·3	44·2	74·3	74·7	67·8	86·6	17·9
Cinnamon	67·3	69·6	84·8	72·2	59·2	100	96·4
Cardamon	45·7	58·9	44·4	41·2	90·2	62·4	82·1
Thyme	75·2	94·5	77·2	100	100	100	100
Tea tree	27·8	43·3	75·4	37·1	68·9	32·7	74·2
Marjoram	0	29·1	14·3	19·8	93·7	12·2	71·1
Ho leaf	47·9	19·2	56·3	5·0	19·0	26·2	31·7
Rosemary	25·5	0·7	53·5	7·6	87·6	2·2	86·4
Peppermint	57·2	42·4	65·9	23·1	76·4	8·0	93·6
Palmarosa	42·2	46·6	63·2	70·9	100	100	100
Lemongrass	66·8	86·2	71·2	42·3	95·5	80·8	0
Sage	24·8	16·9	61·5	16·8	8·2	50·1	0

Oils were solubilized in DMSO before addition to broth cultures.

against *S. pombe*, *S. cerevisiae* and *T. utilis*, cinnamon oil against *S. pombe* and *T. utilis*, cardamon against *S. cerevisiae*, thyme oil against *Staph. aureus*, *S. pombe*, *S. cerevisiae*, *T. utilis* and *C. albicans*, peppermint against *S. pombe*, palmarosa against *S. pombe*, *S. cerevisiae* and *T. utilis*, and lemongrass oil against *S. cerevisiae*. Clove, cinnamon and thyme oils caused $>50\%$ reduction in growth with all organisms. No activity was observed with sage or lemongrass oils against *S. pombe*, and marjoram against *E. coli*.

There was some variation in data from the drop diffusion assay compared with that from the broth dilution assay. For example, the diameter of inhibition in the drop diffusion test for cinnamon oil against *S. cerevisiae* and *T. utilis* was similar at 53 mm and 42·3 mm, whereas in the broth dilution assay, 59% and 100% reduction in cell growth was recorded respectively.

Much higher concentrations of solubilized oils were required for inhibition than with the commercially available preservatives trichlorocarbanilide (TCC), trichlorohydroxydiphenyl ether (Irgasan DP 300) and hexachlorophene, all of which have values of MIC of $<0·1$ ppm (Morris *et al.* 1979). Clove oil in DMSO had a MIC value of 300 ppm against *S. cerevisiae* and *S. pombe* and a MIC value of 400 ppm against *T. utilis*. Cinnamon oil showed a MIC value of 400 ppm against *T. utilis* and *S. pombe*. Thyme showed a MIC value of <300 ppm for all the yeast species and palmarosa oil showed a MIC value of 400 ppm against *S. pombe*, *S. cerevisiae* and *T. utilis*. No MIC was demonstrated for any other oils at the concentrations tested.

The activity of cinnamon oil against *S. cerevisiae* was markedly increased in the absence of DMSO (Table 4). Total inhibition of growth in broth dilution experiments occurred at a concentration of 10 μg ml^{-1} of cinnamon oil, compared with >500 μg ml^{-1} when solubilized in DMSO. These results showed an antagonistic effect of DMSO with cinnamon oil.

Examination of the constituents of the 13 oils shown in Table 3 by reference to published data (Masada 1976), and separations achieved by gas chromatography showed common components which could contribute to the antimicrobial activity of these oils. Such components were identified as eugenol, linalool and *p*-cymene. Figure 1 shows a typical chromatographic separation of cinnamon oil. Eugenol was identified by gas chromatographic separations of oils from *Thymus masticina* (thyme), *Cinnamomum zeylanicum* (cinnamon) and *Eugenia caraphyllata* (clove), respectively. Morris *et al.* (1979) demonstrated MIC values of 500 ppm for pure eugenol against *Staph. aureus*, *E. coli* and *C. albicans*, which correlate closely with the MIC values obtained in this study with intact oils. Linalool, reported previously to have anti-

Table 4 Cell mass of *Saccharomyces cerevisiae* grown with cinnamon oil, 48 h incubation

Oil quantity (μg ml^{-1})	Cell mass (mg)			
	−DMSO		+DMSO	
	Mean	S.D.	Mean	S.D.
0	97·5	6·1	101·7	7·7
10	0		ND	ND
50	0		64	8·5
150	0		26	7·0
500	0		0	

Means and standard deviations (S.D.) are for triplicate experiments.
ND, Not determined.

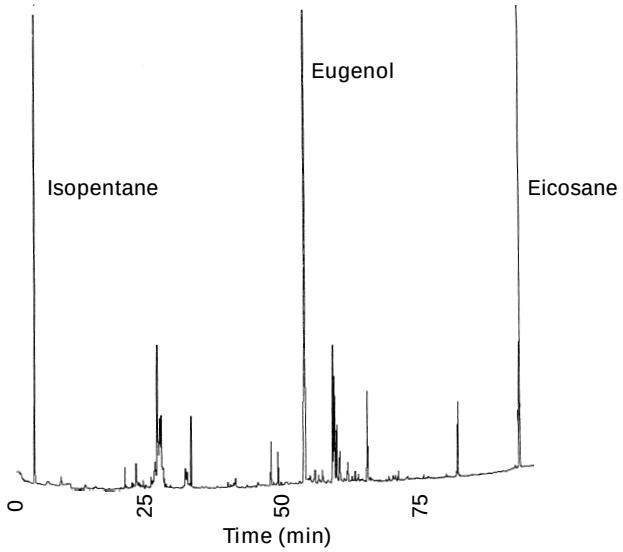

Fig. 1 Separation of components of cinnamon oil by gas chromatography on a BP1 column (50 m × 0·22 mm), with flame ionization detection. Isopentane and eicosane were added to the oil as low and high end markers respectively

bacterial activity (Ross *et al.* 1980) was found in oils of *Salvia officinalis* (sage), *Rosmarinus officinalis* (rosemary), *Thymus masticina* (thyme), *Cymbopogon citratus* (lemongrass), *Couiandrum sativum* (coriander) and *Cinnamomum comphora* (ho leaf) in varying quantities. *p*-Cymene found in *Melaleuca ternifolia* (tea tree), *Thymus vulgaris* and *Couiandrum sativum* oils has antimicrobial activity (Kellner and Kober 1954). α-Pinene has been shown to have activity against *Bacillus subtilis*, *E. coli*, *Staph. aureus*, *Proteus morganii*, *Ps. aeruginosa* and is found in oils of sage, rosemary, tea tree, lemongrass, coriander, thyme, peppermint, and ho leaf (Katayama and Nagai 1960).

Establishing the contribution of these components to the overall antimicrobial activity of any oil would require further investigation. The existence of a synergistic or antagonistic relationship between components may explain the differences in antimicrobial activity that arise for oils of the same species tested in different laboratories. In order to compare results of different studies the precise composition of oils must be known. The geographical origin of oils also needs to be considered in studies of antimicrobial activity. Kowal and Krupinska (1979) found large differences in the MIC values for essential oils of *Thymus pulegioides* from different regions in Poland, and the composition of essential oils from the same species grown in Southern Italy was influenced by the time of harvest (Senatore 1996).

To date very few studies have comparatively examined the effects of oils with and without the use of solubilizer in liquid media. It is essential in such studies that the broth containing the oil is agitated thoroughly in order to achieve full dispersal of the oil, for good reproducibility of data. This study has shown much greater activity (50-fold more) can be attained with cinnamon oil against *S. cerevisiae* in the absence of solubilizer. This may be due to the partitioning of the oil between the aqueous phase and DMSO, distancing the oil from the cells. When no DMSO is used, the oil may be solubilized in the lipid membrane of the organism where it can have a greater effect on cell metabolism.

The mechanism of action of essential oils or their components is unclear, as is the potential for resistance mechanisms arising to their antimicrobial activity. These aspects must be investigated if the use of essential oils as preservatives is to become economically viable.

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