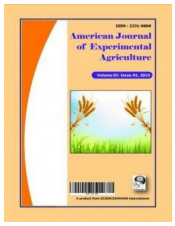

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Eco-friendly Oil-in-Water Emulsion Formulation of Eucalyptus Oil for Controlling Some Important Phytopathogenic Fungi

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Authors' contributions

This work was carried out in collaboration between both authors. Authors TGMM and HAEA performed the research work. Author TGMM wrote the first draft of the manuscript and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Actually, a prodigious interest resides with the improvement and the use of particular biocontrol agent for the control of disease on plants. Our objective was to prepare an alternatives fungicide oil-in-water emulsion of eucalyptus oil and check shelf life stability by measuring number of physical parameters before and after storage tests to be sure that the formulation was stable and evaluates the antifungal activity of the oil itself and its developed formulation oil in water emulsion (EW) by the poisoned food technique. The results revealed that the prepared formulation showed the better storage stability and also, had good antifungal activity as a mycelial growth inhibition against all tested phytopathogenic fungi in compared with the eucalyptus oil. The EC_{50} values of eucalyptus oil on the tested phytopathogenic fungi; *Colletotrichum dematium*, *Drechslera hawaiiensis*, *Humicola fuscoatra*, *Phoma* spp and *Nigrospora sphaerica* were 5025.36, 4440.60, 4625.60, 5277.40 and 6115.79 ppm, respectively and the EC_{50} values of prepared oil water emulsion formulation on the tested phytopathogenic fungi were 196.37, 287.58, 353.00, 312.10 and 490.30 ppm, respectively. Eucalyptus oil could be successfully formulated in the form of stable oil water emulsion. The

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present results demonstrated that oil water emulsion formulation could be used as botanical fungicide to protect some crops from fungal diseases.

Keywords: Oil-in-water; emulsion; formulation; eucalyptus oil; antifungal activity; phytopathogenic fungi.

1. INTRODUCTION

Phytopathogenic fungi are one of the major economic problems of crop production. Apart from their potential to cause losses and fruit decay, many of them represent a very serious risk for consumers because of they produce dangerous mycotoxins [1]. For many years, a variety of different synthetic fungicides have been extensively used as antifungal agents to inhibit the growth of phytopathogenic fungi. However, the widespread use of fungicides has significant drawbacks including increased cost, handling hazards, concern about pesticide residues on food, and threat to human health and environment, in addition; there is a risk of pathogenic microorganisms developing resistance [2,3]. In an attempt to reduce the use of synthetic fungicides, many researchers have concentrated attention on the use of safe and biodegradable alternatives such as natural fungicides to replace synthetic chemicals, for protecting crops from various plant diseases [4-7].

One such alternative is the use of natural plant protectants with pesticidal activity. These natural protectants tend to have low mammalian toxicity, less environmental effects and wide public acceptance. Essential oils (EOs) have become progressively interesting of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-use [4,7] and commonly used as natural preservatives and fragrances in cosmetic products, actually, they was solicited mainly for their antimicrobial properties, new applications as food preservatives [8], growth promoters in livestock [9], antifungal [10,11], natural pesticides in organic agriculture [12,13] and insecticides [14-18] are emerging. EOs typically are volatile and they rapidly evaporate from surfaces. An additional important problem in practical application of essential oil in agriculture is the lake of persistent efficacy in field [19]. It is thus desirable to formulate them, in their effective concentration, in a way that allows minimizing the evaporation and protecting the oil, from high temperature, oxidation and UV light, at the same time. Besides, such formulations should allow for a selective release and for the increase of the shelf life of the oil, minimized evaporation, increased shelf life and, which may increase the biological efficiency [20].

Most agrochemicals are water-insoluble compounds with various physical properties. One of the earliest types of formulations is wettable powders (WP), which are suitable for formulating solid water insoluble compounds that can be produced in a powder form. The second and most familiar type of agrochemical formulation is the emulsifiable concentrates (ECs). These are produced by mixing agrochemical oil with another one such as xylene or trimethylbenzene or a mixture of various hydrocarbon solvents [21].

Recent years have seen a great demand to replace ECs with concentrated aqueous oil-in-water (O/W) emulsions, technically referred to as EWs. Several advantages may be envisaged for such replacements. In the first place, one can replace the added oil with water, which is of course much cheaper and environmentally acceptable. Secondly, removal of the oil could help in reducing undesirable effects such as phytotoxicity, skin irritation, etc. Thirdly, by formulating the pesticide as an O/W emulsion, one can control the droplet size to an optimum value, which may be crucial for biological efficacy. Fourthly, water-soluble surfactants, which may be desirable for biological optimisation, can be added to the aqueous continuous phase. The major advantages of EWs are their relatively low toxicity when compared with ECs, their high flash points and possibility of incorporation of adjuvants in the oil and aqueous phases. In addition, by controlling the droplet size distribution of the oil, one can enhance deposition and spreading and this may increase biological efficiency [21,22].

For this purpose, the formulation of the essential oil in an oil-in-water (O/W) emulsion has been studied in this work. Such formulation can be produced without using toxic or contaminant products and therefore they can be suitable for agricultural applications. Moreover, study the

in vitro antifungal activity against some important phytopathogenic fungi.

2. MATERIALS AND METHODS

2.1 Chemicals

Eucalyptus oil was purchased from Kato Aromatic Company, Giza, Egypt, Non-ionic surfactant; Ethoxylated Castor oil HLB 14.9 (ALKAMULS 14/R) was kindly supplied by Rhodia Company, Milano, Italy. Propylene glycol and sodium hydroxide were purchased from ADWIC; El Nasr Company, Egypt. Calcium carbonate was purchased from Sigma- Aldrich, Germany. Magnesium oxide and methyl red were purchased from Qualikems Fine Chemicals. India. Ammonia Solution was purchased from Prolabo. Agar and Dextrose used in the biological activity test are products of El Nasr Pharmaceutical Chemicals Co., Cairo, Egypt. Streptomycin Antibiotic is a product of El Nil Company Cairo, Egypt. Water used in all preparations obtained from Water distiller LABCONCO water PROT.M PS LABCONCO Corporation, Kansas City, Missouri 64132-USA.

2.2 Fungal Species

Five phytopathogenic fungi were used in this study. They are *Colletotrichum dematium, Drechslera hawaiiensis, Humicola fuscoatra, Phoma spp* and *Nigrospora sphaerica* were provided by Fungicides, Bactericides and Nematicides Department, Central Agricultural Pesticides Laboratory (CAPL), Dokki, Giza, Egypt.

2.3 Emulsion Preparation

The oil-in-water emulsion formulation was prepared by incorporating the oil phase into the water phase; eucalyptus oil (20.00%) was mixed together with the surfactant by stirring (using a magnetic stirrer with hot plate "Terrey Pines Scientific", USA). The water phase was prepared with deionized water, preservative and propylene glycol. Finally, the oil phase was added into the water phase under high shear mixing.

2.4 Physicochemical Characterization

2.4.1 Visual inspection

The concentrated oil water emulsion formulation was observed for color, homogeneity, emulsion stability or separation during storage.

2.4.2 Emulsion stability and re-emulsification [23]

The formulation was diluted at 30°C±2 with CIPAC Standard Waters A and D [24]. In the emulsion characteristics experiment, 5 ml of the formulation sample was separately mixed with standard water: (CIPAC A, 20 ppm hardness, pH 5.00-6.00, Ca^{2+}: Mg^{2+}=1:1 and CIPAC D, 342 ppm hardness, pH 6.00-7.00, Ca^{2+}: Mg^{2+} = 4:1) in a 100 ml measuring cylinder to produce 100 ml of aqueous emulsion. The stopper was placed on the cylinder, which was subsequently turned upside down 10 times. Subsequently, the amount of free oil or cream that separated at the top or the bottom of the emulsion was observed after the emulsion was allowed to stand undisturbed for various time intervals (0.5, 2, 4, 24 h and 24.5 h). For the stability test at low temperature (0°C), 100 ml of each sample was transferred to a glass tube. For cooling, the tube and its content was placed in a refrigerator and remained at 0°C±1 for 7 days. At the end of day 7, the tube was removed from the refrigerator, and allowed to remain undisturbed at room temperature for 3 hrs. The volume of any separated material at the bottom of the tube was subsequently recorded. Accelerated storage procedure was executed by placing the sample (about 50 ml) in a bottle and placing the capped bottle and its contents in an oven at 54°C±2 for 14 days.

2.4.3 Storage stability

Stability tests at elevated temperatures are designed to increase the rate of chemical degradation or physical change of a product. Accelerated testing was performed at elevated temperatures in an attempt to obtain information on the shelf life of a product in a relatively short time. Accelerated testing involves extrapolations from higher to lower temperatures and from shorter to longer storage periods. The Food and Agriculture Organization (FAO) of Pesticides Specifications recommend testing of the relevant product parameters before and after storage at 54°C±2 for 14 days [25]. Also, liquid formulations (oil water emulsion) may be adversely affected by storage at low temperature. Storage at low temperature may result in crystallization of active constituent, significant changes in viscosity or separation of formulation. The liquid formulations should also be tested at 0°C±1 or lower for 7 days [26].

2.4.4 Mean particle size distribution

The mean particle size of oil-in-water emulsion of eucalyptus was determined by light scattering, using Zetasizer Ver.6.20 (Malvern Instruments Ltd., Worcestershire, England) Sample analysis was carried out after sample preparation. The concentrated emulsion was diluted with deionized water (1:100) to avoid multiple scattering effects, and placed directly into a metal jar that circulated the sample through the measuring-glass cuvette.

2.4.5 Freeze -thaw cycles

Freeze-thaw cycles are a method of putting stress on the formulations to stimulate the conditions that are encountered in warehouse storage. Test tube filled with the prepared formulation and hermitically closed was vertically stored for 12 h in freezer at -20°C, and then for 12 h at room temperature 25°C±2. Emulsion was exposed to four such cycles and observed for any physical changes like creaming, coalescence and phase separation. The formulation is considered "stable" if there is no substantial separation after four cycles.

2.4.6 Centrifugation test

Laboratory Centrifuge REMI Centrifuge REMI Equipments Bombay-India- R32A.4000002 was used to determine the stability of the emulsions against gravity. Each sample was centrifuged for 10 min at 5000 rpm in 10 ml graduated plastic test tube, to monitor whether phase separation occurred. The formulation was centrifuged at 25°C.

2.4.7 Persistent foam

Persistent foam is a measure the amount of foam likely to be present in a spray tank or other application equipment following dilution of the product with water. Specified amount of the prepared formulation is added to CIPAC standard waters A and D (95 ml) in the measuring cylinder and made up to the mark. The cylinder is stoppered and inverted 30 times. Stand the cylinder on the bench and left undisturbed for the specified time. The volume of foam was noted [27].

2.4.8 pH measurement

pH value of 1% prepared formulation was measured by using a pH Meter (Jenway model pH 3510). It was recalibrated before testing [28].

2.4.9 Surface tension

Surface tension of the prepared formulation was measured using “Sigma 700” by du Noüy Ring, a platinum/iridium ring. The instrument recalibrated before testing, the sample measured should be clean, homogenous and free from any bubbles and has a stable surface. The surface tension of the prepared formulation was recorded.

2.4.10 Density measurement

Density of the prepared formulation was measured using digital density meter model DDM 2910 by touch screen. Rudolph Research Analytical, USA.

2.4.11 Flash point

Measurement of flash point of the prepared formulation was carried out by tag open-cup method by Koehler instrument company, INC, USA. The flash point was recorded as the temperature at the thermometer when a flash appeared [29].

2.4.12 Viscosity measurement

Viscosity of the prepared formulation was measured at different shear rates, without dilution, using ″Brookfield DV II $^{+}$ PRO″ digital Viscometer. (Brookfield, USA). UL rotational adaptor. The temperature was kept at 25°C during the measurement by water bath TC-502. USA and each reading was taken after equilibrium of the sample. The flow curve of the prepared formulation was obtained by directly reading the viscosity (mPas) and shear rate (s^{-1}) from the viscometer [30].

2.5 Biological Activity

In vitro antifungal activity of eucalyptus oil and prepared stable oil water emulsion formulation was performed using the poisoned food technique [31]. The inhibition zone diameter using potato dextrose agar (PDA) media (200 g grated potato, 20 g dextrose, 15 g agar) [32]. Different concentrations of prepared formulation were used in antifungal activity test against five fungus species viz. *C. dematium, D. hawaiiensis, H. fuscoatra, Phoma* spp and *N. sphaerica*. The Erlenmeyer flasks containing media was sterilized in an autoclave at a pressure of 15 lb/sq inch and temperature 121°C for 15 min. The different concentrations was added to sterilized media, cooled to 30-35°C, and shaken thoroughly. To

avoid bacterial contamination, streptomycin was added to the media before pouring into petri dishes (9 cm diameter). The media were then poured into a set of three petri dishes (three replicates) under aseptic conditions in a laminar flow chamber with filter (Labconco Corporation, Kansas City, Missouri 6432). After partial solidification of the media in the plate, a disc (4 mm diameter) of the fungal species was cut from 1-week-old culture by using a cork borer and inoculated to the center of the poured plates of treatments and control sets. The plates were sealed with Parafilm and then incubated (NÜVE San. Mlztirvetic A.S Ankara, Turkey) at 25°C±2 until the fungal growth in the control dishes was almost completed [12]. We have calculated growth percentage inhibition due to treatment against control, using the following formula [33].

$$\text{Percentage inhibition} = \left(\frac{C - T}{C}\right) x100$$

where C is the average of three replicates of hyphal extension (mm) of control, and T is the average of three replicates of hyphal extension (mm) of plates treated with tested material.

2.6 Statistical Analysis

The concentration of the prepared oil water emulsion formulation that inhibiting the fungi mycelium growth by 50% (EC_{50}) values were determined by the linear regression (LPd line Computer Program) of the probit of the tested fungus percentage inhibition vs. logs the concentrations (ppm) of the tested formulation.

3. RESULTS AND DISCUSSION

3.1 Formulation Characteristics

It is known that emulsions are thermodynamically unstable systems which exist in the metastable state [34]. Emulsion stability refers to the ability of an emulsion to resist changes in its properties over time: the more stable the emulsion, the more slowly its properties change [35]. The instability of an emulsion is often a consequence of two different physical processes: increasing particle size linked with coalescence or flocculation, and migration of particles leading to creaming or sedimentation [36]. The stability of oil water emulsion formulation can be predicted by measuring some physical parameters before and after accelerated tests. Storage at 0°C and 54°C has been used to control physical and chemical stability. It has been generally accepted that two weeks at 54°C represent 2 years in normal conditions. There is no evidence which indicate that a product has a satisfactory shelf life (of at least 2 years) in the different temperature zones. The test thus provides a useful guide for performance after storage in warm or continental temperature climates. However, it is not quite sure that the product which passes these tests will be satisfactory in field conditions [37].

The data in Table 1 showed that the emulsion stability and reemulsification of the prepared eucalyptus oil water emulsion formulation after storage at 0°C±2 for 7 days, 54°C±2 for 14 days and four freeze thaw cycles when diluted at 30°C±2 with CIPAC standard waters A and D produced a white emulsion and no separation or sedimentation and no change in color or appearance through the storage period. Also, the stabilities of the product on initial emulsification and re-emulsification were good. Such signs are good preliminary indication of physical stability. The value of particle size was slightly differed in storage formulation than the fresh prepared formulation. The results showed that particle size distribution of formulation varied from 1.20 µm to 1.28 µm. The droplet size of EW formulations should be below 2.00 microns [38]. Also, no phase separation after centrifugation and four freeze thaw cycles was seen in oil-in-water emulsion samples kept at different storage conditions. The most important parts of chemical stability are performances on accelerated testing and kinetics of pH profiles [39]. The formulation exhibited acidic pH value. The pH values of the prepared formulation were in range (4.58-4.61), indicating that the formulation having acidic character implying that it will have good biological activity [40]. The prepared formulation having the surface tension range (30.13-30.62 mN/m). Lower surface tension is a desirable characteristic for most agricultural sprays because it facilitates the spreading of droplets upon impaction on leaves or other target surfaces, to increase the surface active area and improves penetration and uptake of the product into the plant [41].

The variation of density was 0.9788-1.01896 g/cm^3. The liquid formulation must obtain in this study, was much higher than the prescribed minimum limit of 24.5°C [42]. The prepared formulation in the all storage condition having high value of flash point more than 70°C and this is quite safe. In addition, the viscosity of a fluid is the property that determines the resistance

offered to a shearing force under laminar flow conditions, e.g. resistance to slow stirring, or to flow through a capillary or narrow channel [43]. The prepared formulation of oil water emulsion formulation, showed shear-thinning behavior. The shear thinning fluid viscosity is a function of the shear rate, with higher shear causing lower viscosity (Fig. 1) [44]. Finally, the volume of foam from the samples in CIPAC standard waters A and D is low and passed through the recommended rat of foam (Table 2).

3.2 Formulation Activity

3.2.1 Effect of eucalyptus oil and its oil water emulsion formulation on the linear growth of the tested phytopathogenic fungi

Fungal infections cause significant loss in many economic crops. Crop losses are estimated to be about 14% worldwide [45]. Chemical control may be available to effectively and extensively reduce the effects of most fungal diseases but field application of these chemical fungicides may not always be desirable. Excessive and improper use of these fungicides presents a danger to the health of humans, animals, and the environment. In recent years, interests have been generated in the development of safer antifungal agents from natural plant products such as, essential oils to control fungal diseases. Many essential oils have been reported to have antifungal activities with no side effects on humans and animals [46]. Previous *in vitro* and *in vivo* investigations suggested that the essential oils could be used as effective antifungal agents against many phytopathogenic fungi [47,48].

The *in vitro* antifungal activity of eucalyptus oil and its prepared formulation by PF techniques are reported in Tables 3 and 4 as a minimum effective concentration of 50 % of mycelial growth (EC_{50}) with the corresponding 95% Confidence limits. The results indicated that the percentage inhibition of mycelial growth increases with increasing concentration of eucalyptus oil suggesting that this essential oil inhibited the growth of all tested phytopathogenic fungi in a dose dependent manner. The prepared formulation demonstrated strong mycelial growth inhibition in all tested phytopathogenic fungi; the prepared formulation more effective than the corresponding active material regarding the value of EC_{50} in the case of all examined fungi. The lower the value of EC_{50} is the higher the efficacy of the tested material in the test under consideration. The EC_{50} values of eucalyptus oil on the tested phytopathogenic fungi; *C. dematium, D. hawaiiensis, H. fuscoatra, Phoma* spp and *N. sphaerica* were 5025.36, 4440.60, 4625.60, 5277.40 and 6115.79 ppm, respectively. Whereas, the EC_{50} values of prepared oil water emulsion formulation on the tested phytopathogenic fungi were 196.37, 287.58, 353.00, 312.10 and 490.30 ppm, respectively.

Table 1. Physicochemical properties of oil-in-water emulsion formulation of eucalyptus oil before and after storage

Time		Fresh formulation	After 7 days	After 14 days	Freeze-thaw
Temperature		**Room temp.**	**0°C**	**54°C**	**4 Cycles**
pH value (1%)		4.61	4.58	4.59	4.60
Surface tension (mN/m)		30.13	30.40	30.39	30.62
Density(g/cm^3)		0.9788	0.9991	1.0189	1.0007
Flash point (°C)		Over 70°C	Over 70°C	Over 70°C	Over 70°C
Particle size distribution (mean diameter)		1.28 µm	1.25 µm	1.23 µm	1.20 µm
Stability of emulsion and re-emulsification	0.5 h	0/0	0/0	0/0	0/0
	2 h	0/0	0/0	0/0	0/0
	4h	0/0	0/0	0/0	0/0
	24h	0/0	0/0	0/0	0/0
	*REE	0/0	0/0	0/0	0/0

**REE: Re-emulsification after 24 h (24.5)*

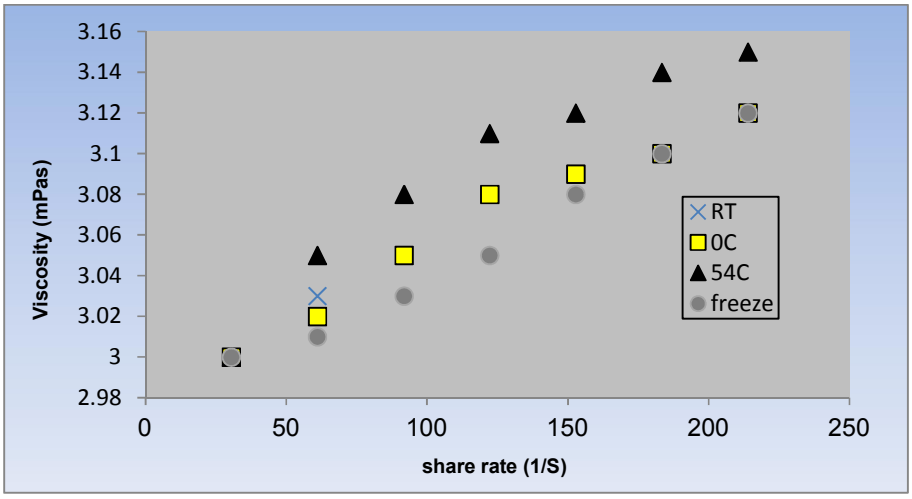

Fig. 1. Rheological properties of oil-in-water emulsion formulation of eucalyptus oil before and after storage

RT: room temperature; 0°C: 7 days at 0°C 54°C: 14 days at 54°C±2; Freeze: four freeze thaw cycles

Table 2. Volume of persistence foam (cm^3) observed in oil-in-water emulsion formulation of eucalyptus oil before and after storage test in CIPAC Waters A and D

Sample code	CIPAC water A			CIPAC water D		
	1 min	5 min	12 min	1 min	5 min	12 min
Fresh formulation	5	4	3	4	3	3
After 7 days 0°C	6	5	4	5	4	3
After 14 days 54°C	5	5	4	5	4	3
Freeze-thaw cycles	6	5	4	4	3	4

Table 3. Effect of different concentrations of active substance of eucalyptus oil against different phytopathogenic fungi

Tested Fungi	% inhibition at different concentrations (ppm)						EC_{50}	95 % Confidence limit	
	2000	4000	6000	8000	10000	12000		Lower	Upper
C. dematium	20.84	42.83	56.21	65.90	77.79	87.85	5025.3	4238.6	5814.7
D. hawaiiensis	15.21	44.65	65.09	77.58	85.21	89.98	4440.6	2703.6	5979.9
H. fuscoatra	14.47	27.71	62.88	75.56	83.50	88.58	4625.6	2741.2	6333.5
***Phoma* spp**	8.34	34.65	57.25	72.33	81.83	87.91	5277.4	3201.7	7338.6
N. sphaerica	2.38	22.59	48.65	68.29	80.82	88.39	6115.7	2982.0	9438.9

Table 4. Effect of different concentrations of oil water emulsion formulation of eucalyptus oil against different phytopathogenic fungi

Tested Fungi	% inhibition at different concentrations (ppm)					EC_{50}	95 % Confidence limit	
	62.5	125	250	500	1000		Lower	Upper
C. dematium	7.78	28.77	61.77	87.68	97.82	196.37	159.7	239.4
D. hawaiiensis	0.17	3.45	24.05	65.88	93.61	287.58	237.6	664.4
H. fuscoatra	1.05	8.32	32.28	67.87	91.74	353.00	171.6	849.7
***Phoma* spp**	0.44	6.78	35.87	77.88	97.12	312.1	239.1	409.4
N. sphaerica	2.08	8.82	25.26	50.77	75.96	490.30	352.5	769.7

4. CONCLUSION

The oil water emulsion formulation is free from any solvent except water, in addition to dilution tolerance, and using of environmentally friendly oils. Emulsion formulation was prepared. Characterization of emulsion for its pH, surface tension, viscosity, flash point, density, indicated a stable formulation, which was safe to handle and transport. Further, stability of emulsion was established at various process parameters (temperature, centrifugation and free-thaw properties). The formulation had good antifungal activity as a mycelial growth inhibition against all tested phytopathogenic fungi in compared with eucalyptus oil. The presented results are initial findings and evaluation of the prepared formulation should be continued in the field to determine its efficacy, and to estimate the economic aspects of its use and compare the activity with commercial fungicide.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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