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Formulation and Evaluation of Synergistic Effect of Garlic oil and D-Limonene Nanoemulsion for its Anti-fungal Properties against Tomato leaf spot disease

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ABSTRACT

Garlic oils have promising possibilities for a wide range of applications in the food and pharmaceutical industries. Their widespread utilization is limited as they are lipophilic and highly volatile. Furthermore, they also possess strong odor and low physicochemical stability. Therefore, the present study aims to investigate the characteristics of garlic oil nano-emulsion through investigating its antifungal activity. The optimized nanoemulsion of D-Limonene and Garlic oil using 75% water and 20% emulsifiers having particle size range of 10 to 12nm showed significant better antifungal activity against Tomato leaf spot disease without loss of antioxidant potential in comparison to Garlic oil and D-Limonene as individual nanoemulsion. We have formulated Garlic oil, D-Limonene and Synergistic combination of both based nanoemulsion with antipest and antifungal properties. It is stabilized and particle size characterized by using Malvern zeta sizer has been tested using and found the size in the range of 10.0 to 25.0nm. It was further evaluated in field for its antifungal activity.

Keywords: Garlic oil, D-Limonene nanoemulsion,
Anti-fungal properties, Tomato leaf spot disease.

INTRODUCTION

Nano emulsions demand a large amount of energy to prepare due to their thermodynamically unstable structures. Mainly, two methods are used for the preparation of Nano emulsions,[1] High-energy methods, which includes intense shear stirring, ultrasonic generator, and high-pressure homogenizers, and [2]Micro-fluidizer, which employ high mechanical energy to combine and crush the oil and water phase. Nanoemulsion is a type of formulation with similar size in the range between 10 and 100nm[3]. They maintain optical transparency, have high stability against gravitational separation and droplet aggregation due to their tiny particles size, and also can improve the bio-efficacy when compared to traditional micro emulsions (>200 nm)[4,5]. It has been observed that nanoemulsions also require a lower concentration of surfactants, generally, 5-15% according to traditional microemulsion, which require at least 20% surfactants. The use of organic solvents and adjuvants are avoided in food, drinks agrochemicals, and medications[6]. There are many low-energy methods reported with high specific reaction conditions such as high temperature, constant compositions, high workup reaction procedure etc.[7].

Natural products are widely used as biologically active compounds and also considered as an alternative for sustainable insect pest management in agriculture. *Essential oils* are important agent and very helpful in crop protection due to its medicinal properties. Garlic *(Allium sativum)* belongs to the Alliaceae family and commonly consumed in food ingredient and scented culinary herb, native to the high plains of Western Central Asia and cultivated throughout the world[8].

The *Essential oil* of Garlic is obtained by steam distillation of Garlic[9]. The Sulphur containing constitutes diallyl disulfide and diallyl trisulfide are the predominant constituent of the oil, with diallyl disulfide comprising up to 60% of the oil by weight. Garlic is one of many plants that discovered to create secondary metabolites as a defense to pests and disease damage[10]. Garlic plant is broadly utilized for food flavoring and widely hailed to have a positive impact on human health well-being, based on its characteristic organo-sulfur mixes.Specifically, *Essential oils* extracted from garlic *(Allium sativum L)* are found to present with advantageous anti-carcinogenic, anti-diabetic, and anti-microbial activity, which is found to be significant for a host of medicinal and pharmacologic applications.

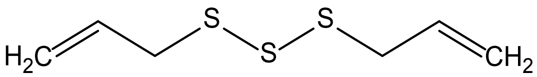

Fig. 1. Structural Formula of Garlic Oil

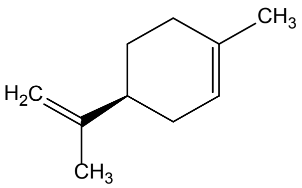

Fig. 2 Structural Formula of D-Limonene

D-Limonene is a common natural compound which is commonly found in many vegetables, citrus fruits and in some spices with citrus scent and lemon-like flavor. Chemically this monoterpene exhibit toxicity against several insects [11]. It is widely used in pesticides products, cosmetic products (soaps and perfumes) and in food products. It is also used as an insecticide, insect repellent and animals (dogs and cats) repellent and solvent or fragrance in pesticide formulations. It is commonly used in products such as certain food, soaps and perfumes. According to food and drug administration (FDA), D-Limonene recognized as safe as a flavoring ingredients. However, research on nanoemulsion is growing, but nanoemulsion based on a plant extract oils are rarely explored due to their weak water solubility. Encapsulation of oils in oil-in-water emulsions or nanoemulsions can alleviate this problem[12]. The current study aims to generate nanoemulsions based on Garlic oil and D-Limonene utilizing a low energy spontaneous emulsification approach, which is the first report of its sort to our knowledge. In this report nanoemulsion is developed by mixed surfactants (both anionic and non-anionic like calcium dodecylbenzene sulfonate, Tristrylphenol ethoxylate sulfate and castor oil ethoxylate 40 moles) using garlic oil as oil phase and activities.

It is reported that when compared to single pure surfactants, blended surfactants have been shown to have superior applicability[13]. Therefore, we used the mixture of non-ionic Octylphenol ethoxylate (PO-10), Castor oil ethoxylate 40 moles (Stepantex CO-40), EO-PO copolymer, Tween 80 and anionic calcium dodecylbenzene sulfonate (CABS), Tristrylphenol ethoxylate sulfate (Soprophor series), to prepare nanoemulsion formulation.

Garlic oil used as a botanical insecticide because of its medicinal properties, its structural formula is illustrated in Fig. 1, however its water insolubility limits its use use in pest management. In our research, we used the low-energy method, which involves adding water to an oil-surfactant mixture at a specific temperature and with different compositions via different ratios of different anionic and non-ionic surfactants during emulsion process to obtain an oil-in-water (O/W) nanoemulsion[14]. The use of nanotechnology in this context is highly significant due to its ability to manipulate, characterize or fabricate materials or devices, which have dimensions of around 1 to 100 nm. Nano-emulsions are typically possessing dimension below 500nm and have enhanced assimilation properties achieved through the use of mucosa. It demonstrates a high level of stability in suspension and is produced through the use of high-energy or low-energy methods due to their highly reduced dimensions. We investigated different parameters to get the best emulsification conditions for the preparations of nanoemulsion of Garlic oil and D-Limonene by using PIC method. The emulsion stability at ambient and long term stability at 54ºC for 2 weeks was studied to check the shelf life and stability of emulsion and PH effect and also the insecticidal activity was further studied.

EXPERIMENTAL

Materials

Garlic oil and D-Limonene were purchased from local market. Octylphenol ethoxylate (PO-10), Castor oil ethoxylate 40 moles (Stepantex CO-40), EO-PO copolymer,Tween 80 and anionic calcium dodecylbenzene sulfonate (CABS), Tristrylphenol ethoxylate sulfate (Soprophor series) were received from Solvay, Croda, Stephan and Core Chemicals. All the chemicals used are of analytical grade. The Stirrer, Overhead stirrer (IKA Model Euro-ST 20D S000) and distilled water, centrifuge, PH Meter, beaker, measuring cylinder, Particle size Analyzer (Malvern), laminar flow, Mettler Toledo weighing balance were used for the preparation of nanoemulsion and further studies.

Preparation of Primary nanoemulsion of Garlic oil (GNE)

The primary nanoemulsion was formulated using a spontaneous emulsification process[15]. The surfactants and the Garlic oil were mixed in different ratios keeping Garlic oil as 5%. Then this reaction mixture slowly added into aqueous phase and stirred it for 2 h at room temperature. Different proportion of surfactants mixed with 5% Garlic oil and water (15:5:5, 10:10:5, 5:15:5, 3:12:5) were standardized.

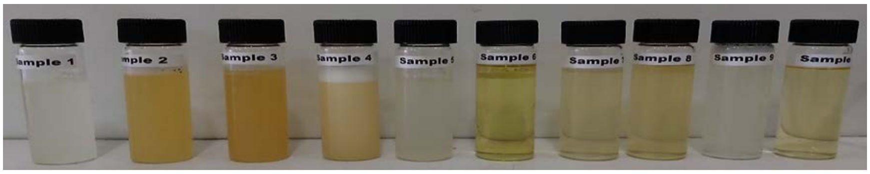

Fig. 3. Garlic oil nanoemulsion with different percentage of surfactants

Preparation of Primary nanoemulsion of D-Limonene (DLNE)

In the same way, nanoemulsion of D-Limonene was formulated using surfactants mix and water with 5% D-Limonene (15:5:5, 10:10:5, 5:15:5, 3:12:5, 3:15:5) were standardized.

Fig. 4. D-Limonene nanoemulsion with different percentage of surfactants

Preparation of Primary nanoemulsion of D-Limonene with Garlic oil

Standardized process for primary emulsion of D-Limonene, surfactant mix and water (10:10:5) was used to prepare Nanoemulsion with a 5% concentration of Garlic oil by a similar procedure of spontaneous emulsification. Garlic oil is added to the nanoemulsion of D-Limonene oil was added drop wise to the standardized primary nanoemulsion of D-Limonene and stirred it using IKA overhead stirrer at 500rpm for 2 h at room temperature.

Determination of particle size of nanoemulsions

The Malvern particle size analyzer uses Dynamic Light Scattering (DLS) to detect changes in scattering intensity caused by Brownian motion of a particle in suspension or emulsion. The DLS measurement was carried out at 25ºC. The sample is prepared in distilled water. 5 mL of the sample solution is taken into vial.

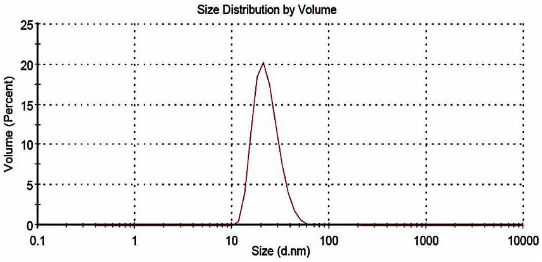

Fig. 5. Droplet size of GNE 6

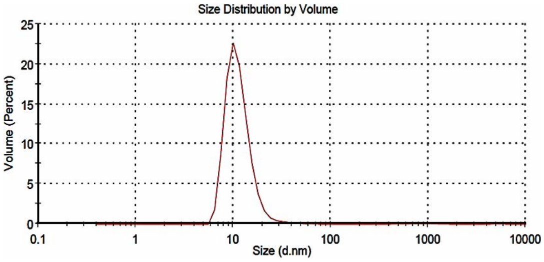

Fig. 6. Droplet size of DLNE

Table 1: Average droplet size (nm) of Garlic Oil Nanoemulsion (GNE)

Nanoemulsion	GNE1	GNE2	GNE3	GNE4	GNE5	GNE6	GNE7	GNE8	GNE9	GNE10
Composition	CABS-10 +FFT 40-5+5 Garlic oil +water	CABS-15 +FFT 40-5+5 Garlic oil +water	CABS-20 +FFT 40-5+5 Garlic oil +water	Soprophor FLK-15 +FFT 40-5+5 Garlic oil +water	Soprophor BSU-15 +FFT 40-5+5 Garlic oil +water	Soprophor 4D384-10 +FFT 40-10+5 Garlic oil +water	Soprophor 4D384-5 +FFT 40-15+5 Garlic oil +water	Soprophor 4D384-3 +FFT 40-12+5 Garlic oil +water	Soprophor 4D384-3 +FFT 40-15+5 Garlic oil +water	Soprophor 4D384-3 +FFT 40-17+5 Garlic oil +water
Size (nm)	25.15 ±3.5	25.05 ±3.0	25.25 ±3.5	24.95 ±3.2	25.15 ±3.5	23.39 ±2.5	23.15 ±3.4	24.82 ±3.5	25.15 ±4.5	29.25 ±4.5

Table 2: Average droplet size (nm) of D Limonene Nanoemulsion (DLNE)

Nanoemulsion	DLNE1	DLNE2	DLNE3	DLNE4	DLNE5	DLNE6	DLNE7	DLNE8	DLNE9	DLNE10
Composition	CABS-10 +FFT 40-5+5 D-Limonene +water	CABS-15 +FFT 40-5+5 D-Limonene +water	CABS-20 +FFT 40-5+5 D-Limonene +water	Soprophor FLK-15 +FFT 40-5+5 D-Limonene +water	Soprophor BSU-15 +FFT 40-5+5 D-Limonene +water	Soprophor 4D384-10 +FFT 40-10+5 D-Limonene +water	Soprophor 4D384-5 +FFT 40-15+5 D-Limonene +water	Soprophor 4D384-3 +FFT 40-12+5 D-Limonene +water	Soprophor 4D384-3 +FFT 40-15+5 D-Limonene +water	Soprophor 4D384-3 +FFT 40-17+5 D-Limonene +water
Size (nm)	10.99±3.5	9.83±3.0	11.94±3.5	11.05±3.2	10.38±3.5	11.71±2.5	10.63±3.4	10.83±3.5	12.54±4.5	12.07±4.5

Table 3: Average droplet size (nm) of D Limonene & Garlic oil Nanoemulsion (DLGNE)

Nanoemulsion	DLGNE1	DLGNE2	DLGNE3	DLGNE4	DLGNE5	DLGNE6	DLGNE7	DLGNE8	DLGNE9	DLGNE10
Composition	CABS-10 +FFT 40-5 +2.5 D-Limonene +2.5 Garlic oil +water	CABS-15 +FFT 40-5 +2.5 D-Limonene +2.5 Garlic oil +water	CABS-20 +FFT 40-5 +2.5 D-Limonene +2.5 Garlic oil +wataer	Soprophor FLK-15 +FFT 40-5 +2.5 D-Limonene +2.5 Garlic oil +water	Soprophor BSU-15 +FFT 40-5 +2.5 D-Limonene +2.5 Garlic oil +water	Soprophor 4D384-10 +FFT 40-10 +2.5 D-Limonene +2.5 Garlic oil +water	Soprophor 4D384-5 +FFT 40-15 +2.5 D-Limonene +2.5 Garlic oil +water	Soprophor 4D384-3 +FFT 40-12 +2.5 D-Limonene +2.5 Garlic oil +water	Soprophor 4D384-3 +FFT 40-15 +2.5 D-Limonene +2.5 Garlic oil +water	Soprophor 4D384-3 +FFT 40-17 +2.5 D-Limonene +2.5 Garlic oil +water
Size(nm)	10.99±3.5	9.83±3.0	11.94±3.5	11.05±3.2	10.38±3.5	11.71±2.5	10.63±3.4	10.83±3.5	12.54±4.5	12.07±4.5

Determination of morphology of nanoemulsions

Transmition Electron Microscopy (TEM) is used to examine the morphology of the Garlic oil and D-Limonene nanoemulsion. A drop of nanoemulsions is deposited on a 400 mesh carbon-coated copper grid and allowed to dry in vacuum[16], then rinsed with distilled water and stained with negative stain (2% of uranyl acetate). The excess water is carefully dried using filter paper before being examined under TEM at various magnification settings.

Table 4: The PH of Garlic oil and D-Limonene nanoemulsions was determined using PH meter at 25±1°C

No.	Garlic Oil Nanoemulsion (GNE)	pH	No.	D-Limonene Nanoemulsion (DLNE)	pH
1	GNE 1	5.3	1	DLNE 1	5.6
2	GNE 2	5.3	2	DLNE 2	5.8
3	GNE 3	5.5	3	DLNE 3	5.7
4	GNE 4	5.5	4	DLNE 4	5.6
5	GNE 5	5.6	5	DLNE 5	5.7
6	GNE 6	6.1	6	DLNE 6	6.9
7	GNE 7	6.1	7	DLNE 7	6.8
8	GNE 8	6.2	8	DLNE 8	6.8
9	GNE 9	6.2	9	DLNE 9	6.9
10	GNE 10	5.9	10	DLNE 10	6.8

Determination of PH of nanoemulsions

Determination of stability of nanoemulsions

The strength of Garlic oil and D-Limonene nanoemulsion were checked by centrifuging the nanoemulsion at 3500rpm for 30 min[17] and also studied at 54°C for 2 weeks.

RESULTS AND DISCUSSIONS

Preparation of primary emulsion and nanoemulsion

Garlic oil, CABS, castor oil ethoxylate 40 moles and Soprophor 4D384 with distilled water are used as raw materials without any purification. PH and ionic strength have less of an influence on FFT-40 surfactant. For the manufacture of the (O/W) main emulsion, D-Limonene oil, castor oil ethoxylate 40 moles (FFT 40), and soprophor 4D384 are used along with distilled water. After experimenting with various raw material combinations, it was discovered that the optimal ratio of oil, surfactants, and distilled water is 10:10:5. When surfactant is increased to Garlic oil or D-Limonene oil, the droplet size decreases (Fig. 3 & 4). The combination of these three emulsifiers improves stability and achieves the desired particle size.

Droplet size of Garlic oil and D-Limonene Oil nanoemulsions

The droplet size of Garlic oil nanoemulsion is shown in Table 1 and Fig. 3 is shown in Table 1 and Fig. 3. Droplet size of Garlic oil nanoemulsion with surfactants ranged from 23.39±2.5 nm to 29.25± 4.5nm Fig. 5. The prepared nanoemulsions with 5% Garlic oil, 10% Soprophor 4D384 and FFT-40 10% (GNE 6) and showed the droplet size and is found to be 23.39±2.5nm. It has been observed that when the concentration of surfactant is increased in nanoemulsion resulted the turbidity and droplet size of nanoemulsion is decreases[18].

Droplet size of D-Limonene oil nano-emulsion is shown in Table 2 and Fig.4. The average droplet size of D-Limonene oil nanoemulsion with surfactants ranged from 9.83±3.0nm to 12.54±4.5nm Fig. 6. The prepared nanoemulsions with 5% D-Limonene oil, 10% Soprophor 4D384 and FFT-40 10% (DLNE 6) and 5% D-Limonene oil, 5% Soprophor 4D384 and FFT-40 15% (DLNE 7) and the minimum droplet size are found to be 11.71± 2.5nm and 10.63±3.4nm.

Stability of nanoemulsions

The stability of nanoemulsion depends on the depends on the physicochemical characteristics of its ingredients including surfactants, oil phase and water phase[19]. This study was carried out by centrifuging the nanoemulsions (Garlic oil and D-Limonene) at 3500rpm for 30 minute. Nanoemulsions are physically stable at room temperature and after stability study at 54°C for 14 days (Fig. 3, 4 and 7). The stability of nanoemulsions droplets of the oil was due to the surfactant which reduced the interfacial free-energy providing the mechanical barrier to calescence[20].

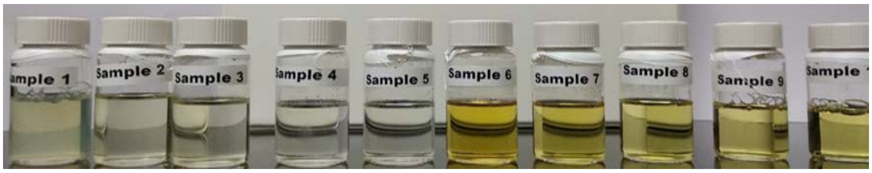

Fig. 7. Stability of nanoemulsion

PH of nanoemulsions

pH have an important role in stability of the nanoemulsion. The PH of Garlic oil and D-Limonene oil's nanoemulsions measured given in the Table 4. The PH value of the Garlic oil nanoemulsion ranged from 5.3 to 6.2 and that of the D-Limonene nanoemulsions from 5.6 to 6.9. No relation has been observed for the PH change with increasing concentration of surfactants in nanoemulsions.

In this study, we report nanoemulsion of garlic oil and D-Limonene using mixed surfactants (non-ionic and anionic) through the low-energy emulsification method. On the basis of Malvern Zeta sizer, the nanoemulsions were obtained in the average size range of 10nm for Garlic oil and 25.01nm for D-Limonene. The nanoemulsion was further evaluated in vitro for antioxidant, IGR, insect antifeedant, antifungal, and nematicidal activity.

Biological examples

The synergistic pesticide action of the inventive mixtures can be demonstrated by the experiments below. Where the action of a combination (ready-mix) of active ingredients is greater than the sum of the actions of each of the components, a synergistic effect exists. In the field of agriculture, the term "synergy" is commonly understood to mean "calculation of the synergetic and antagonistic responses of herbicide combinations" as defined by Colby S. R. published in the journal Weeds, 15,p.[20], incorporated herein by reference in its entirety. For a particular combination of two active components, the predicted activity may be computed as follows;

Colby's formula for calculating synergism between two active ingredients

$$E = X + Y - \frac{XY}{100}$$

Where, E= Expected % control by mixture/combination of Compound A and Compound B in a defined dose
X= Observed % control by Compound A
Y= Observed % control by Compound B

$$\text{Ratio} = \frac{\text{Observed value (\% control)}}{\text{Expected value (\% control)}}$$

Ratio of O/E > 1, means synergism observed

Field Experiment

The field bio efficacy of ready mix formulation, tank mix and individual of Garlic extract nanoemulsion and D-Limonene extract nanoemulsion has been carried to judge the efficacy against Leaf spot (early blight) diseases of tomato.

Details of Experiment

a) Experiment design : Randomized Block Design
b) Replication : Three
c) Treatments : Nine
d) Plot size : 30 sq. m.
e) Spacing : 100 cm x 50 cm
f) Crop : Tomato
g) Time of application : At fruiting stage, 80 days after transplanting
h) Spray Volume : 500 l/h
i) Method of application : Foliar spray with knap sack sprayer

The tomato nursery was raised and transplanted in the main field at row to row 100 cm and plant to plant 50 cm spacing. Rest of the agronomic practices like fertilizer applications, weeding, irrigation (drip irrigation) were as per the standard agronomic practices. The spraying was done at 80 days after transplanting with the help of knapsack sprayer. The observations on severity and incidence of various diseases and fruit counts were taken as follow:

Leaf spot disease (caused by Alternaria solani):

Observations recorded at 7 and 14 days after spraying by observing 100 leaves per plot. The disease severity recorded by following 0-9 scale described as below;

Grade	Percent leaf area infected
0	No symptoms
1	1-10%
3	11-25%
5	26-50%
7	51-75%
9	>75%

Leaf spot (severity) =______Sum of numerical disease rating x 100
Total no. of samples x maximum of disease rating scale

%Disease control=100-% leaf spot disease severity in treatment x 100
%leaf spot disease severity in untreated

%Disease control used and Colby's formula applied to judge the synergism as per the formula give above.

Table 5: Treatment details for field studies

Sr. No	Treatment details with application rate (ml per liter)
1	Garlic extract 5%+D-Limonene extract 5% Nano emulsion (Ready mix) @ 5 ml/l
2	Garlic extract 2.5%+ D-Limonene extract 2.5% Nano emulsion (Ready mix) @ 5 ml/l
3	Garlic extract 5% Nano emulsion @ 5 ml/l+ D-Limonene extract 5% Nano emulsion (tank mix)
4	Garlic extract 2.5% Nano emulsion+ D-Limonene extract 2.5% Nano emulsion (tank mix)
5	Garlic extract 5% Nano emulsion @ 5 ml/l
6	Garlic extract 2.5% Nano emulsion @ 5 ml/l
7	D-Limonene extract 5% Nano emulsion @ 5ml/l
8	D-Limonene extract 5% Nano emulsion @ 5ml/l
9	UTC (Untreated Check)

Table 6: Bio efficacy against tomato leaf spot

Sr. No	%Leaf spot control						
	7 DAA			14 DAA			
	Obs. Value	Cal. Value	Colby/s Ratio O/E	Obs. Value	Cal. Value	Colby/s Ratio O/E	Synergism (Y/N)
1	99.6	86.65	1.15	96.7	81.94	1.18	Y
2	92.4	75.58	1.22	93.5	67.01	1.4	Y
3	88.2	86.65	1.02	57.2	56.8	1.01	Y
4	77.4	75.58	1.02	42.6	41.4	1.03	Y
5	64.2			58.2			
6	52.4			43.7			
7	62.7			56.8			
8	48.7			41.4			
9	0			0			

Colby ratio higher means strong synergism

The bio efficacy results of field trials show that ready mix formulation of Garlic extract and D-Limonene extract Nano emulsion formulation gave very high level of synergism and provides excellent control of tomato leaf spot disease at 7 and 14 days after application.

The tank mix of Garlic extract and D-Limonene extract also show low level of synergism at different concentrations. Treatment number 1 and 2 shows very high level of synergism compared to treatment number 3 and 4.

Before Treatment

After Treatment

CONCLUSION

The optimized nanoemulsion of D-Limonene and Garlic oil showed significant better antifungal activity without loss of antioxidant potential in comparison to Garlic oil. This system has the potential for agriculture application.

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Conflict of interest

The author declare that we have no conflict of interest.

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