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A RAPID HPTLC METHOD FOR IDENTIFICATION AND QUANTIFICATION OF THYMOL IN AN AYURVEDIC FORMULATIONS

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ABSTRACT

Background: Hakam, Erand pak, Ajmodadi, Pathyadi are very popular Ayurvedic (churna) medicines practiced in India; however, unfortunately, they possess several quality control issues. **Objective:** The aim of this study was to find out a simple, accurate and sensitive HPTLC method for the detection and quantification of marker molecule, thymol on these Ayurvedic formulations for standardization. **Materials and methods:** Methanolic extraction (reflux) was performed from the above four churnas as well as crude drug (*Trachyspermum ammi*). HPTLC was done using thymol as a standard. The mobile phase was a mixture of toluene-ethyl acetate (93:7, v/v) and detection at 254 and 366nm. **Results:** The Rf was detected at 0.58. Thymol was quantified in crude drug (*Trachyspermum ammi*) is 13.34%. **Conclusion:** This method can be successfully employed for standardization and quantitative analysis of thymol in Ayurvedic formulations (churnas) and also be helpful to clinicians and pharmacists to draw significant role of thymol present in all these samples.

KEYWORDS: HPTLC, Ajwain, Thymol, Churna, Ayurveda.

1 INTRODUCTION

According to World Health Organization (WHO); traditional, complementary, alternative, or non-conventional medicines are used by 70-95% of global population particularly in developing countries for their healthcare.[1] Moreover, the use of herbal medicines has increased remarkably in line with the global trend of people returning to natural therapies. The growing use of botanicals (drug and other products derived from plants) by the public is forcing moves to assess the health claims of these agents and to develop standards of quality and manufacture.[2] Herbal formulations have achieved widespread acceptability as therapeutic agents for several chronic diseases such as Pain, Constipation, diabetes, arthritis, liver diseases, gastrointestinal disorders, cough and cold, memory loss and immunodeficiency. These medicines are readily available in the market from health food stores without prescriptions and have been widely used in India, China, USA, and have fairly good market all over the world.[3] The validation of herbal products is a major public health concern both in developed and resource-poor countries, where fakers sell adulterated herbal medicines.[4] It is feasible that the introduction of scientific validation would control the production of impure or poor quality herbal products and would eventually ensure their rational use.[5]

Standardization of herbal medicines is the process of prescribing a set of standards or inherent characteristics, constant parameters, definitive qualitative and quantitative values that carry an assurance of quality, efficacy, safety and reproducibility.[6] A herbal product cannot be considered scientifically valid if the drug tested has not been authenticated and characterized in order to ensure reproducibility in the manufacturing of the product. Moreover, many dangerous and lethal side effects have recently been reported, including direct toxic effects, allergic reactions, effects from contaminants, and interactions with herbal drugs.[7] On this background, standardization is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and manufacturing of a herbal drug.[8] The Indian system of medicine, mainly comprising of Ayurveda, Siddha and Unani, is one of the oldest holistic management system with thoroughly documented remedies. Ayurveda, a part of cultural heritage of India, is widely respected for its uniqueness and global acceptance as it offers natural ways to treat diseases and promote healthcare.[9] Unfortunately, standardization and quality control have remained grey areas in the preparation of Ayurvedic medicines. Till date, most of the ayurvedic formulations are lacking in their defined quality control parameters and method of its evaluation.[10]

In Ayurveda, different powder (churna) formulations such as Hakam churna, Ajmodadi churna, Erand pak churna, Pathyadi churna, Trikatu churna, Sitopaladi churna, Hingavastaka churna, Avipattikara churna,

Sringyadi churna and Talisadya churna are most commonly used from ancient times to treat pain, constipation, asthma, cough and cold, tuberculosis, fever, indigestion, chronic rhinitis/sinusitis and other inflammatory and respiratory disorders. Interestingly, it has been noted that in all these formulations have one of the most essential herbal ingredients such as Trachyspermum ammi (ajwain), which is responsible for hepatoprotective activity, Antilithiasis and diuretic activity, Antiplatelet-Aggregatory, anti-inflammatory, anti-tussive effects, anthelmintic activity, antioxidant properties, etc, as mentioned in Ayurvedic Formulary of India.[11]

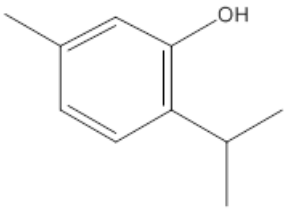


Fig. 1: Chemical structure of Thymol.

2. MATERIALS & METHODS

2.1 Plant material and Test Samples

Fruit of Trachyspermum ammi (ajwain) were procured from the local market of Moga, Punjab, India, and Ayurvedic powdered formulations such as Hakam churna, Ajmodadi churna, Erand pak churna, Pathyadi churna were obtained from Ramakrishna Ayurvedic store, Moga, Punjab, India (Table 1). All these powdered formulations were prepared following the instructions as mentioned in the Ayurvedic Formulary of India. All the raw botanicals as well as churna formulations were authenticated by Dr Alok Sharma, Head of Department of Pharmacognosy, ISF College of Pharmacy. The physicochemical parameters for quality standards were evaluated for each raw material and Ayurvedic formulations as mentioned in Ayurvedic Pharmacopoeia of India (Table 2).

2.2 Collection of herbal material

Ajwain *(Trachyspermum ammi)* and ayurvedic formulation procured from local market moga, Punjab.

2.3 Chemicals and reagents

TLC plates coated with silica gel $60F_{254}$ for HPTLC were purchased from Merck, Germany and Thymol was purchased from Sigma, USA. All other chemicals, reagents and solvents were used are of AR grade.

2.4 Preparation of test samples

5 g of drug and churna samples were taken separately, refluxed with 25 ml of methanol for 2hr, filtered through Whatmann filter paper no. 41 and this procedure was repeated thrice. The pooled filtrate was concentrated and volume was adjusted to 10 ml with methanol in a volumetric flask. Aliquot of each extract was further diluted 50% for quantification by HPTLC.

3 Instrumentation and chromatographic condition

A Camag HPTLC system comprising of Linomate V automatic sample applicator with Camag TLC Scanner 3 and Camag WinCAT software were used for detection and quantification of thymol in the single herbs and Ayurvedic formulations. The standard solutions and test samples were spotted in the form of bands (8 mm bandwidth) with 100 ml Hamilton syringe on pre-coated silica gel plates (Merck, $60F_{254}$, 10-10 cm) using Camag Linomate V applicator. The plates developed upto 80 mm with a solvent system Toluene: Ethyl acetate (93:7) in Camag glass twin-trough chamber previously saturated with mobile phase vapor for 30 min at 25 °C. The densitometric scanning was performed on Camag TLC Scanner 3 at absorbance 366 nm (deuterium lamp, slit dimension 5.0-0.45 mm) and operated by multilevel WinCATS planar chromatography manager software (Table 4). Spots were well resolved in the chromatogram of extracts of samples from single herbs or Ayurvedic powder formulations, and the spot of standard thymol was at 0.58. The amount of thymol present in the samples was calculated using calibration curve of standard thymol and expressed as mg/g of dry samples. The experiments were repeated thrice to confirm results (Figure 2).

4 Crude drug validation

The proposed method was validated in terms of linearity, pre-cision, accuracy, robustness, limit of detection (LOD), and limit of quantitation (LOQ) according to the International Conference on Harmonization guideline (Table 3).

4.1 Linearity

From a standard working solution of Thymol (1 µg/mL), 2.0 µl, corresponding to the concentration of thymol 100-700 ng/band, were separately applied on the HPTLC plate. The calibration curves were obtained by plotting between the peak areas versus the concentration of standard thymol.

4.2 Precision

For the study of repeatability and intermediate precision, three concentrations of standard solution including 0, 10, 20, 30, and 500 ng/band were applied on the HPTLC plate (n = 3). Repeatability was determined by analysis of thymol at three different time intervals within one day, while the intermediate precision was determined on three consecutive days using the proposed method. The precision was expressed as percentage of relative standard deviation (% RSD).

4.3 Accuracy

Accuracy of the method was confirmed by measurement of recovery. Three different concentrations of thymol (approximately 97%, 101%, and 97% of the pre-quantified extract) were added to the ajwain extract. Samples were prepared in triplicate. Data were calculated and expressed as percentage of recovery as follows.

44.4 Limit of detection and limit of quantitation

The limit of detection (LOD) is the lowest level of analyte that can be detected in a sample, but not necessarily quantified, under the stated experimental conditions. The limit of quantification (LOQ) was identified as the lowest amount of analyte that can be detected and quantified with acceptable accuracy, precision, and variability.

LOD = 0.35 (SD/ *S)* and LOQ = 1.77 (SD/ *S)*.

4.5 Robustness

The chromatographic conditions were slightly modified for the robustness test of thymol (2.0 µg/band). The time from spotting of thymol on the HPTLC plate to development. Mobile phase composition was toluene: ethyl acetate (93:7). The % RSD of the peak areas of thymol reference standard was calculated for robustness variations.

Table 1: Details of sample.

Sample	Brand Name	Batch No	Quantity
Sample A	Planet Ayurveda	04	100gm
Sample B	Baidyanath	118.P.006	100gm
Sample C	Patanjali	#A-AJC049	100gm
Sample D	__	__	500gm
Sample E	Planet Ayurveda	214.P.070	100gm

Table 2: Quality parameters of Ayurvedic drugs and Ayurvedic formulations.

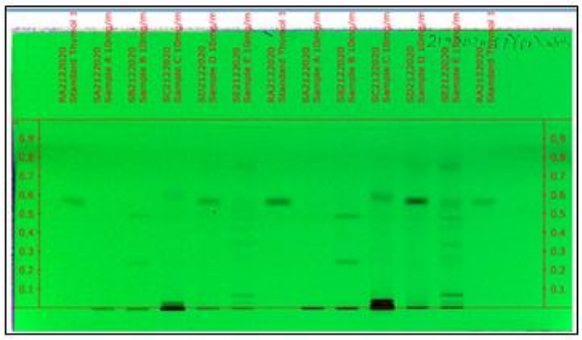
S. No	Sample	Loss on drying (%)	Total ash (%)	Acid insoluble ash (%)	Water soluble ash (%)	Extractive value (%)	pH
1	Hakam churna	5.61	5.09	4	2.25	11.5	5.54
2	Erand pak churna	1.30	1.65	0	0.75	9	6.18
3	Ajmodadi churnas	4.79	8.9	7	2.75	38	4.81
4	Ajwain	7.28	8.34	8	1.75	21	5.78
5	Pathyadi churna	4	4.5	3.5	3	36	5.61

Table 3: Method validation parameter of sample D.

S.No	Validation parameter	Result of sample D
1	Percentage of thymol	13.34 %
2	Limit of detection	0.35 ng
3	Limit of quantification	1.07 ng
4	Accuracy	98.77 %
5	Linearity range	100-700 ng

Table 4: Chromatographic profiles of Standard and Samples by HPTLC Method.

S.No	Sample(10mg/ml)	Sample ID	Solvent system	Detection	Rf
1	A	SA2122020	Toluene: Ethyl Acetate (93:7)	366 nm	NF
2	B	SB2122020	Toluene: Ethyl Acetate (93:7)	366 nm	NF
3	C	SC2122020	Toluene: Ethyl Acetate D(93:7)	366 nm	NF
4	D	SD2122020	Toluene: Ethyl Acetate (93:7)	366 nm	0.59
5	E	SE2122020	Toluene: Ethyl Acetate (93:7)	366 nm	NF
6	Standard-Thymol	RA2122020	Toluene: Ethyl Acetate (93:7)	366 nm	0.58



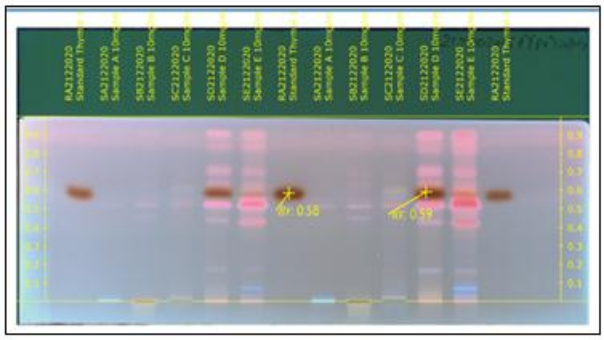


Figure 1: HPTLC Fingerprinting at 254nm. Figure: 2 HPTLC Fingerprinting at 366nm.

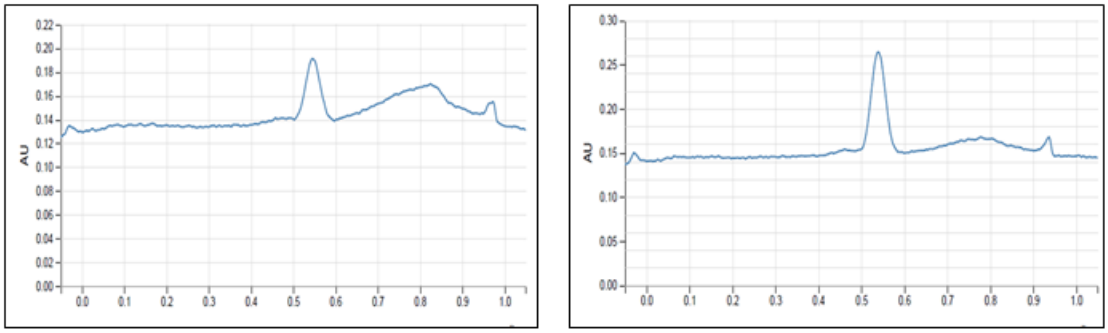


Figure 3: TLC Chromatogram of standard marker Thymol developed using mobile phase Toluene: Ethyl acetate (93:7 v/v) different concentration, T-1 (2.0 µl) and T-2 (5.0 µl).

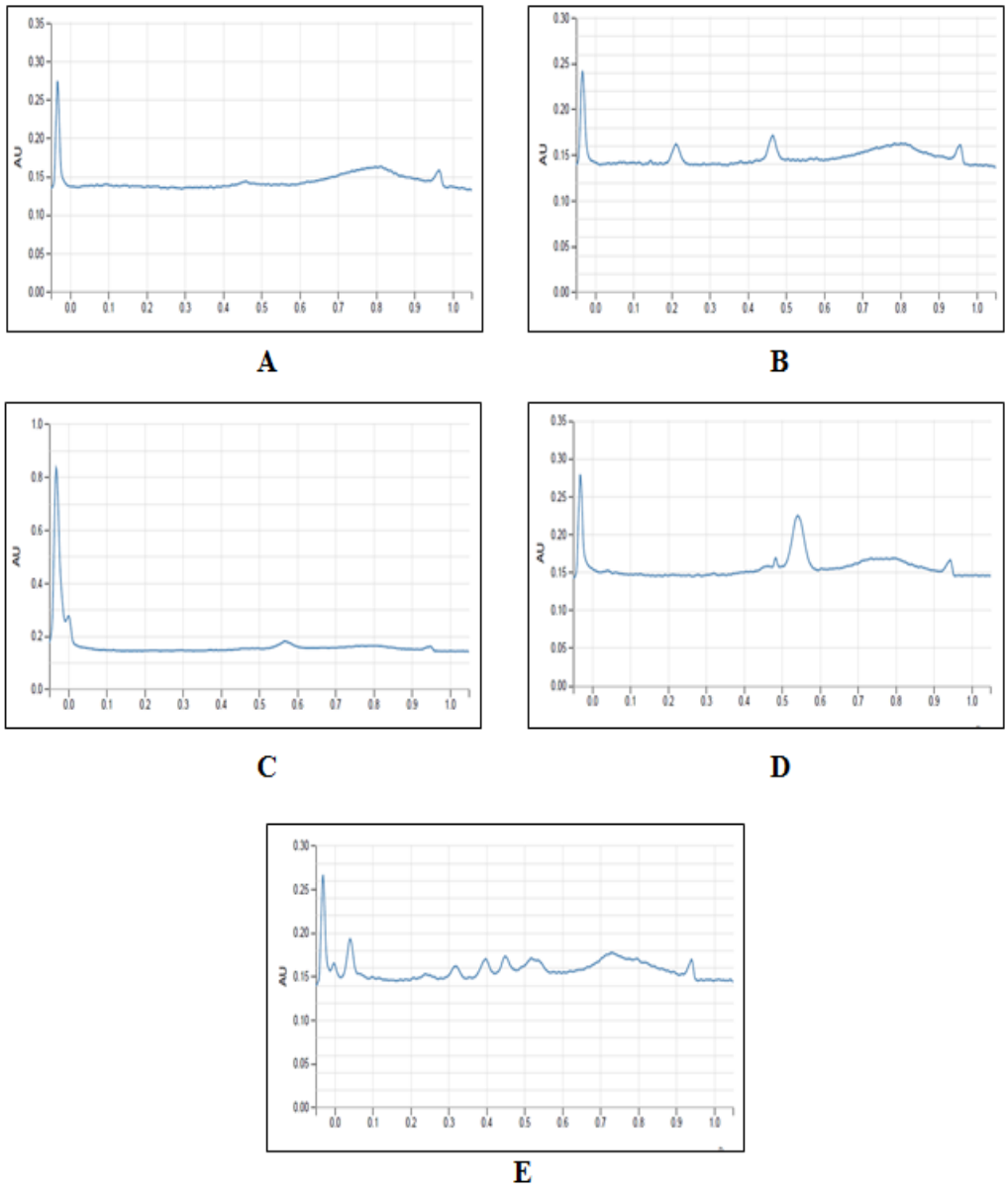


Figure 4: TLC Chromatogram of following samples developed using mobile phase Toluene: Ethyl acetate (93:7 v/v) with 2.0 µl concentration.

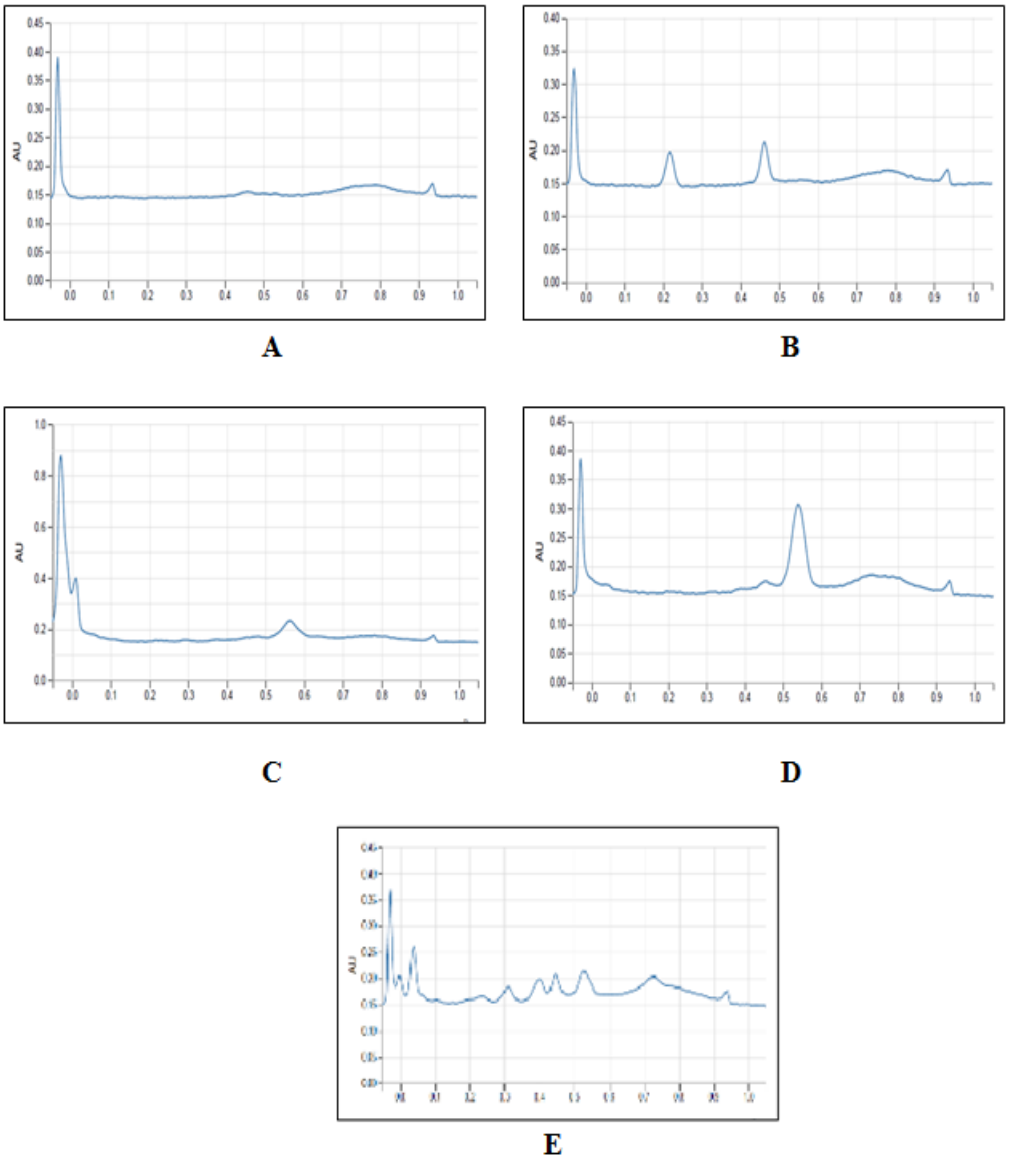


Fig. 5: TLC Chromatogram of samples developed using mobile phase Toluene: Ethyl acetate (93:7 v/v) with 5.0 µl.

5. RESULTS AND DISCUSSION

Trachyspermum ammi Sprague has been widely used in Ayurveda as a medicine. This is commonly called as Ajwain from family Apiaceae or Umbelliferae. This is a native of Egypt. It is cultivated in Afghanistan Iraq, Iran, Pakistan and India. In India it is grown in West Bengal, Rajasthan, Uttar Pradesh, Gujarat, Maharashtra, Bihar and Madhya Pradesh. The seeds have 2.0-4.4% brown colored oil. The main active component is thymol which is useful in the treatment of lack of appetite, bronchial problems and gastro intestinal troubles. This is anti-spasmodic, germicidal and even fungicidal. Thymol has application in toothpastes and even in perfumery. The health benefits are mainly because of various phytochemicals. This highlights the importance of this untapped resource in removing against various human ailments.

The HPTLC procedure was optimized with a view to develop a stability indicating assay method. The solvent system of the mobile phase having toluene: ethyl acetate (93:7, v/v) gave dense, compact and well separated spots of the single herbal ingredients as also Ayurvedic formulation at 254nm & 366nm (Fig. 1 & 2). The limit detection for piperine and the limit of quantification was found to be 0.35ng and 1.07ng respectively. These values are considered to be good enough for a reasonable accuracy in most of the laboratories worldwide. Moreover, thymol concentration was found 13.34 %. The assay values were found to be within the standard acceptable limits and so the method can be adopted for estimation of thymol in Ayurvedic formulations. The peak area and concentration was subjected to least square linear regression analysis to calculate the calibration equation $y=1.25x10^{-8}+6.417x10^{-4}$ and regression coefficient (r2) was 0.997871.

The TLC Chromatogram of standard marker Thymol which was identified using mobile phase Toluene: Ethyl acetate (93:7 v/v) with two different concentrations i.e. 2.0 µl and 5.0 µl illustrate in figure 1 and figure 2. Further, the Rf value of standard thymol and crude drug (*Trachyspermum ammi*) were observed at 0.58 and 0.59 which are describe in Table 4, whereas figure 1 and 2 showed fingerprinting profile of different samples by using Toluene: Ethyl acetate (93:7 v/v) as a mobile phase at 2.0 µl and 5 µl concentrations.

Linearity studies were carried out and there exists linearity in the concentration range of 100ng – 700ng for thymol. The good average recovery values obtained in recovery studies indicate that the proposed method is accurate for estimation of drug in Ayurvedic powder (churna) formulation. Thus, the developed method was found to be accurate, precise, suitable and cost effective for the estimation of thymol in Ayurvedic formulations and crude drug (*Trachyspermum ammi*).

CONCLUSION

This HPTLC method can be successfully employed for standardization and quantitative analysis of thymol in Ayurvedic formulations (churnas) as well as raw materials and also be helpful to clinicians and pharmacists to draw significant role of thymol present in all these samples.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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