



Invitro Evaluation & Antimicrobial Activity of Citrullus Species (C. colocynthis and C. lanatus)

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ABSTRACT

Background: Citrullus Species (C. colocynthis & C. lanatus) commonly belong to the Cucurbitaceae family. Traditionally, Citrullus plants have been used for a diverse range of therapeutic activities like antibacterial, larvicidal, antilipidemic, etc. On reviewing the literature available online; it was visible that evaluation of antimicrobial activity of Citrullus species based on geographical variation has not been carried out till date.

Purpose: This paper seeks to comprehensively discuss and evaluate the antimicrobial action of Citrullus species from various geographical locations of India, such as Rajasthan, Punjab, and Haryana.

Methods: Soxhlet extraction technique was utilized to prepare the extracts as per authentic & standard guidelines. The obtained fractions were then subjected to preliminary phytochemical screening studies, followed by evaluation of antimicrobial action using a well-established Cup and Plate Method.

Results: Higher antimicrobial activity was observed for ethyl acetate extract, as compared to the acetone and methanol extract. It was also seen that the antimicrobial activity of the plants from Rajasthan surpassed the samples collected from Punjab and Haryana. The phytochemical screening studies involving ethanol and ethyl acetate fractions depicted the existence of phytoconstituents like tannins, flavonoids and quercetin.

Conclusions: The antibacterial activity of fractions of C. Colocynthis and C. Lanatus fruits was found to be more potent for the ethyl acetate extract against Escherichia coli as compared to Staphylococcus aureus. The existence of compounds such as tannins, flavonoids etc., in the extracts, may be the reason for the scientifically documented pharmacological profile of different Citrullus species.



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1. Introduction

India is a country that has been endowed with a wide array of medicinal plants, and many of them have the potential to exert therapeutic action. Herbs and medicinal plants act as natural remedies to treat and manage health conditions safely and efficaciously (Kolipaka, Singh, Kumar, Venkata, & Sai, 2023). The use of herbal remedies is increasing at an alarming rate owing to their low degree of side effects, wide applicability, and cost-efficient nature. They contain innumerable phytoconstituents and bio-actives such as tannins, alkaloids, triterpenoids, flavonoids, etc., which can be found in different parts of a plant such as leaves, blossoms, roots, fruit etc. These phytoconstituents have been explored for a wide range of activities including antioxidant, gastroprotective, anthelmintic, anticancer, demulcent, antidiuretic action etc.

Citrullus Species (C. colocynthis & C. lanatus) are types of flowering plants that belong to the Cucurbitaceae family. C. colocynthis is a plant native to Asia, and is known to have bitter flavoured fruits, known as 'bitter apple' or 'tumba' (Hamid & Mehmannaavaz, 2015). C. Lanatus originates from South Africa and is a type of the most commonly found melon. These species of Citrullus are an important and indispensable part of the Ayurveda and have both nutritive and therapeutic benefits (Deshmukh, Jain, & Tambe, 2015). Traditionally, Citrullus plants have been used for a diverse range of therapeutic activities like antibacterial, larvicidal, antilipidemic, etc. They have also found their use as an anti-inflammatory, anticancer, antioxidant, anti-giardial and, analgesic agent. The major utility of these plants lie in their antibacterial (Thirunavukkarasu, Ramanathan, Ravichandran, & Ramkumar, 2010; W, IJ, & S, 2012), prosthetic hyperplasia, and anti-secretary activity (Oluwole,

Balogun, & Temitope, 2013). The seeds of *Citrullus* Species contain phytoconstituents such as vitamins, alkaloids, flavonoids, amino acids, cardiac glycosides, terpenoids, carotenoids, etc. The different amino acids found in *Citrullus* plants include arginine, glutamine, and aspartic Acid.

Microbes or microorganisms are minuscule creatures that are present in abundance and are responsible for the precipitation of microbial diseases. Based on this fact, the anti-microbial uses of *Citrullus* caught the attention of researchers. On reviewing the literature available online, it was visible that evaluation of antimicrobial activity of *Citrullus* species based on geographical variation has not been carried out till date. This paper aims to comprehensively discuss and evaluate the antimicrobial activity of *Citrullus* species from various geographical locations of India, such as Rajasthan, Punjab, and Haryana.

2. Materials and Methods

2.1. Authentication of Plant Materials

Exhaustive dry fruits of *Citrullus* species were procured from wild regions of Rajasthan, Punjab, and Haryana. Before commencing the research work, the botanical identity of fruits was confirmed by the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab, India.

2.2. Chemicals, Reagents, And Solvents

Chemicals, reagents, and solvents of the laboratory and analytical grade were procured from E Merck, Delhi, India, and S.D. The chemicals utilized for extraction and phytochemical studies were obtained from Fine Chemicals, Mumbai, India. The analytical grade chemicals, reagents, and solvents of E Merck, Delhi, India, and S.D.

2.3. Preparation of Various Extracts of *Citrullus* Species

The powdered plant materials were extracted separately and successfully in a soxhlet apparatus using solvents in increasing order of their polarity viz. petroleum ether, acetone, ethanol, and water.

The marcs of both the plants were separately extracted with water, by heating on a hot plate for 2 hours by following the decoction process.

The bioactive extracts of each plant material were suspended uniformly in water, placed in a round bottom flask, and partitioned successfully and separately using the solvent ethyl acetate by heating at 50°C for 30 minutes along with continuous stirring to obtain their ethyl acetate

fraction. A similar process was followed to obtain the extracts or fractions of *Citrullus* species in different solvents.

2.4. Fluorescence analysis of *Citrullus* Fruits

The fluorescence analysis was carried out for both the species of *Citrullus*. The results of the same have been mentioned in Table 1 and Table 2.

Table 1: Fluorescence analysis of *C. Colocynthis* fruits.

S. No.	Drug treatment with reagent	Visible (Day Light)	UV Light 254nm (Long)	UV Light 366nm (Long)
1	Without reagent	Light Green	Light Brown	Dark Brown
2	Water	Light Green	Light Brown	Reddish Brown
3	95% alcohol	Dark Green	Dark Green	Reddish Black
4	Aqueous Extract	Reddish Brown	Blackish	Blackish
5	Conc. Sulphuric Acid	Blackish Brown	Blackish Brown	Blackish Brown
6	Conc. Hydrochloric Acid	Blackish	Blackish Green	Reddish Black
7	Glacial Acetic Acid	Brownish	Brownish Green	Brownish
8	Chloroform	Dark Green	Greenish Black	Reddish Black
9	Dil. Ammonia Solution	Dark Green	Brownish Black	Brownish Black
10	Ferric Chloride Solution	Blackish Green	Blackish	Reddish Black

Table 2: Fluorescence analysis of *C. lanatus* fruits.

S. No.	Drug treatment With reagent	Visible (Day Light)	UV Light 254nm (Long)	UV Light 366nm (Long)
1	Without reagent	Light Green	Light Brown	Dark Brown
2	Water	Light Green	Light Brown	Brownish
3	95% alcohol	Dark Green	Dark Green	Reddish Black
4	Aqueous Extract	Reddish Brown	Greenish Black	Blackish Black

5	Conc. Sulphuric Acid	Blackish Brown	Greenish Black	Blackish Black
6	Conc. Hydrochloric Acid	Blackish Brown	Blackish Green	Reddish Black
7	Glacial Acetic Acid	Brownish	Brownish Green	Blackish
8	Chloroform	Light Green	Greenish Brown	Reddish Black
9	Dil. Ammonia Solution	Light Green	Brownish Black	Brownish Black
10	Ferric Chloride Solution	Blackish Green	Blackish Brown	Reddish Black

2.5. Antimicrobial studies of fruits of *Citrullus Species* (*C. colocynthis* and *C. lanatus*)

2.5.1. Antibacterial study

Staphylococcus aureus and Escherichia coli bacterial strains were used in the present research work and were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh.

2.5.2. Method of Testing

Inoculation of Staphylococcus aureus and Escherichia coli was carried out in a conical flask having 100 sterile nutrient broth. The conical flasks were then incubated for a time period of 24 hours at a temperature of 37°C. This was known as seeded broth.

2.5.3 Standardization of Seeded Broth (Viable Count)

A millilitre of each bacteria's seeded broth was diluted by using 99 millilitres of sterile water containing a 0.05 percentage of Tween 80 (8 drops of Tween 80 were added to 100 ml of normal saline). 1 ml of this preparation was taken and diluted to 10 ml by using sterile water. This step was repeated to obtain different dilutions of the seeded broth, ranging from 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and reaching up to 10^{-10} . To carry out the study further, 0.2 ml of each of the prepared dilutions was inoculated onto a solidified agar nutrient medium in a Petri plate by using the spreading method. The number of colonies formed on the petri plates were counted and observed after an incubation period of 24 hours at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The seeded broth was then appropriately diluted to contain between 10^{-6} to 10^{-7} cfu (colony forming units) per ml. This was then referred to be a working stock which was used for *in vitro* anti-bacterial studies (seeded broth).

2.5.4 Preparation of Solution of Test Drug

Dimethyl sulfoxide (DMSO) was then utilized to prepare the drug solution in a specific gravity bottle. One hour prior to its use, DMSO was uncrated from the refrigerator to allow it to reach room temperature. Solutions of test drugs (extracts/fractions at concentrations of 20 mg/ml in DMSO) and standard drug (Streptomycin sulphate) 100 mg/ml in DMSO were prepared. DMSO was kept under solvent control throughout the conduct of the experiment.

2.5.5 Preparation of Culture Media

Nutrient broth and agar media were used for bacterial growth. The culture media were prepared and then sterilized by using an autoclave at 15 lb/sq. inch pressure at 120°C for a time span of 20 minutes.

2.5.5.1 Composition of Agar Media

To prepare nutrient agar media, 28 gms of nutrient Agar (Hi-Media) was added to 1000 ml of distilled water. The pH was then adjusted to 7.4 ± 0.2 . The constituents of the agar media along with their quantities have been represented in Table 3.

Table 3: Components of Nutrient Agar Media.

Constituent	Quantity (gms/litre)
Peptone	5
Sodium Chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15

2.5.5.2 Composition of Nutrient Broth

The Nutrient broth was prepared by adding 1gm of nutrient broth (Hi-media) to 1000 ml of distilled water. The pH was adjusted to 7.4 ± 0.2 . The constituents of the nutrient broth along with their quantities have been represented in Table 4.

Table 4: Components of Nutrient Broth.

Constituent	Quantity (gms/litre)
Peptone	5
Sodium Chloride	5
Beef extract	1.5
Yeast extract	1.5

2.5.6. Determination of Antibacterial Susceptibility of the Test by Cup-Plate Method

The antimicrobial activity of crude extracts of *Citrullus* species can be evaluated based on the Agar well diffusion

assay, also known as the Cup and Plate method. This method is dependent on drug diffusion from a cavity, through and across a solidified layer of agar in a Petri dish, such that multiplication of the added microorganisms is inhibited in a circular zone in the vicinity of the drug-containing cavity. The petri plates containing solidified nutrient agar media were inoculated with 0.2 ml of seeded broths containing 10^6 to 10^7 cfu per ml test microorganisms with the help of a micropipette, and then using a glass spreader. The next step involved the creation of four wells in the Agar layer with the help of an aluminium borer. 0.2 ml of the test drugs solution at 20 mg/ml concentration was added into two of the wells. The entire procedure was performed in maintained aseptic conditions. The petri plates were then left undisturbed for 1 hour at room temperature to allow the solution to diffuse into the medium. This was followed by incubating the plates for a time period of 24 hours at $37 \pm 1^\circ\text{C}$. Once the incubation was complete, the mean diameter (in millimetres) was noted to determine the zone of inhibition.

2.5.7. Determination of Minimum Inhibitory Concentration (MIC)

Due to the occurrence of a significant zone of inhibition in the above study, a follow-up step to determine the minimum inhibitory concentration (MIC) of test drugs was planned and carried out. The various extracts and fractions of *C. Colocynthis* and *C.lanatus* fruits were studied for their antibacterial activity by determining the MIC using a two-fold serial dilution approach.

The hydroalcoholic extracts were separately dissolved in DMSO to obtain a 10 mg/ml solution. Six assay tubes were used for each strain. To obtain the first dilution, 1.8 ml seeded broth, along with 0.2 ml of test drug were added and mixed in an assay tube. 1 ml of the seeded broth was then added to each of the remaining five assay tubes. 1 ml of the content was pipetted out from the first assay tube into the second assay tube and mixed thoroughly to obtain the second dilution and so on till six such dilutions were obtained (1000 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$, 250 $\mu\text{g/ml}$, 125 $\mu\text{g/ml}$, 62.5 $\mu\text{g/ml}$, and 31.25 $\mu\text{g/ml}$). The experiment was performed in aseptic condition. Similarly, the solvent control (DMSO) solutions were prepared in seeded broth such that the dilution contained the amount of DMSO as that of the previous dilutions.

The assay tubes were then put into an incubator for 24 hours at $37^\circ\text{C} \pm 1^\circ\text{C}$. The observations were made at the end of 24 hours. The assay tubes were then taken out of the incubator and observed for any deposits, and shaking the tubes for aerating the solution and suspending any bacteria that may have collected at the bottom of the test tubes.

The MIC (in $\mu\text{g/ml}$) was then determined for both the test and standard drug by choosing the lowest concentration that led to an apparent complete inhibition of microbial growth. The solvent control tubes were also checked for any inhibitory influence of DMSO. Antibacterial activity of various fractions of *C. Colocynthis* and *C.lanatus* fruits are presented in tabular data.

2.5.8. Evaluation of In Vitro Antimicrobial Activity

The various crude extracts of *Citrullus* species in solvents such as acetone, methanol extracts, and ethyl acetate, obtained from different regions were subjected to evaluation of antimicrobial activity using well-established procedures known as Cup Plate Method. The results determined from the present work are represented in form of MIC. The experiment was performed under aseptic conditions by cup & plate method.

3. Results and Discussion

3.1. Antibacterial Activity

Acetone, methanol, and ethyl acetate fractions obtained from *Citrullus colocynthis* and *Citrullus lanatus* of wild regions of Rajasthan, Punjab, and Haryana were evaluated for their in vitro antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* by using cup plate method. It was observed that the ethyl acetate fractions depicted a higher degree of antibacterial activity in comparison to the standard drug streptomycin opposite *Staphylococcus aureus* and *Escherichia coli*.

3.2. Antibacterial Activity against *Staphylococcus Aureus*

It was observed that ethyl acetate *Citrullus colocynthis* fraction collected from Rajasthan (MIC 250 $\mu\text{g/ml}$, Zone of inhibition reading 20.70 mm), Punjab (MIC 250 $\mu\text{g/ml}$, Zone of inhibition reading 20.60 mm) and Haryana (MIC 250 $\mu\text{g/ml}$, Zone of inhibition reading 20.01 mm) showed close antibacterial profile, as compared to standard antibacterial drug streptomycin (MIC 125 $\mu\text{g/ml}$, Zone of inhibition reading 28.44 mm).

Similar pattern of antibacterial activity against *Staphylococcus aureus* was observed for the extracts of *Citrullus lanatus*, where ethyl acetate fraction of the fruits gathered from Rajasthan (MIC 500 $\mu\text{g/ml}$, Zone of inhibition reading 19.89 mm), Punjab (MIC 500 $\mu\text{g/ml}$, Zone of inhibition reading 19.65 mm) and Haryana (MIC 500 $\mu\text{g/ml}$, Zone of inhibition reading 19.45 mm) showed a close antibacterial profile, as compared to standard

antibacterial drug streptomycin (MIC 125 µg/ml, Zone of inhibition reading 28.44 mm).

Finally, the results of the antibacterial activity against *Staphylococcus aureus* confirmed that *Citrullus colocynthis* fruits exhibited strong antibacterial activity against *Staphylococcus aureus* than *Citrullus lanatus* fruits. Results of the antibacterial activity are depicted in Table 5.

Table 5: Antibacterial activity of various extracts and ethyl acetate fractions from methanol extract of *C. colocynthis* and *C. lanatus* obtained from Rajasthan, Punjab and Haryana against *Staphylococcus aureus*.

Name of Bacteria	Microbial activity / Zone of inhibition reading (mm)					
	<i>C. colocynthis</i> / <i>C. lanatus</i>					
	31.25 (µg/ml)	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)	1000 (µg/ml)
Control (Negative)	---	---	---	---	---	---
Streptomycin (Positive)	24.58	25.87	28.44	28.50	28.65	28.70
Acetone (Rajasthan)	---	---	---	5.60 / 4.40	5.62 / 4.41	5.65 / 4.45
Acetone (Punjab)	---	---	---	5.25 / 4.27	5.27 / 4.32	5.32 / 4.35
Acetone (Haryana)	---	---	---	5.15 / 4.14	5.16 / 4.18	5.18 / 4.21
Ethanol (Rajasthan)	14.52 / 11.85	15.44 / 12.50	16.87 / 13.55	18.57 / 15.58	18.60 / 15.60	18.65 / 15.75
Ethanol (Punjab)	14.25 / 10.85	15.10 / 11.25	16.20 / 12.35	16.89 / 14.55	17.45 / 14.60	17.50 / 14.62
Ethanol (Haryana)	13.45 / 10.25	14.50 / 11.10	15.75 / 12.10	16.50 / 14.25	17.30 / 14.28	17.31 / 14.31
Ethyl acetate fraction (Rajasthan)	17.58 / 16.50	19.45 / 18.19	20.47 / 18.78	20.70 / 19.15	20.72 / 19.65	20.75 / 19.70
sEthyl acetate fraction (Punjab)	17.29 / 16.50	19.31 / 18.19	20.30 / 18.78	20.60 / 19.15	20.65 / 19.65	20.69 / 19.70

3.3. Antibacterial Activity against *Escherichia Coli*

It was observed that the ethyl acetate fraction of *C. Colocynthis* gathered from Rajasthan (MIC 125 µg/ml, Zone of inhibition reading 13.60 mm), Punjab (MIC 125 µg/ml, Zone of inhibition reading 13.45 mm) and Haryana (MIC 125 µg/ml, Zone of inhibition reading 13.10 mm) showed close antibacterial profile against *E. coli*, as compared to standard antibacterial drug streptomycin (MIC 125 µg/ml, Zone of inhibition reading 17.58 mm).

Similar pattern of antibacterial activity against *E. Coli* was observed in *C.lanatus* fruits where, only ethyl acetate extract of *C.lanatus* fruits collected from Rajasthan

(MIC 125 µg/ml, Zone of inhibition reading 11.05 mm), Punjab (MIC 125 µg/ml, Zone of inhibition reading 10.88 mm) and Haryana (MIC 125 µg/ml, Zone of inhibition reading 10.50 mm) showed close antibacterial profile against *E. coli*, as compared to standard antibacterial drug streptomycin (MIC 125 µg/ml, Zone of inhibition reading 17.58 mm).

Finally, the results of the antibacterial activity against *E. coli* confirmed that *C. colocynthis* fruits exhibited stronger antibacterial activity against *E. coli* than *C. lanatus* fruits. The results of the antibacterial activity are depicted in Table 6.

Table 6: Antibacterial activity of various extracts and ethyl acetate fraction obtained from methanol extract of *C. colocynthis* and *C. lanatus* fruits obtained from Rajasthan, Punjab and Haryana against *Escherichia coli*.

Name of Bacteria	Microbial activity / Zone of inhibition reading (mm)					
	<i>C. colocynthis</i> / <i>C. lanatus</i>					
	31.25 (µg/ml)	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)	1000 (µg/ml)
Control (Negative)	---	---	---	---	---	---
Streptomycin (Positive)	15.75	16.58	17.58	17.60	17.75	17.80
Acetone (Rajasthan)	---	---	---	---	3.24 / 2.45	3.25 / 2.50
Acetone (Punjab)	---	---	---	---	3.15 / 2.22	3.16 / 2.25
Acetone (Haryana)	---	---	---	---	2.95 / 2.08	2.96 / 2.13
Ethanol (Rajasthan)	---	---	10.14 / 8.58	11.25 / 9.25	12.58 / 9.89	12.60 / 9.90
Ethanol (Punjab)	---	---	10.01 / 8.35	11.11 / 9.09	12.45 / 9.60	12.47 / 9.55
Ethanol (Haryana)	---	---	9.89 / 8.05	11.01 / 8.99	12.15 / 9.11	12.16 / 9.12
Ethyl acetate fraction (Rajasthan)	---	12.45 / 10.24	13.60 / 10.88	13.65 / 11.01	13.69 / 11.03	13.70 / 11.07
Ethyl acetate fraction (Punjab)	---	10.18	10.88	11.01	11.03	11.07
Ethyl acetate fraction (Haryana)	---	12.10 / 10.01	13.10 / 10.50	13.15 / 10.55	13.16 / 10.57	13.20 / 10.60

Finally, the results of antibacterial action determined against *Staphylococcus aureus* and *Escherichia coli* of fractions of *C. colocynthis* and *C.lanatus* gathered from wild regions of Rajasthan, Punjab, and Haryana confirmed that only ethyl acetate fraction of tested samples possess potent activity against *E. coli* bacterial strain than *Staphylococcus aureus* bacterial strain. The results have been depicted in Figure 1.

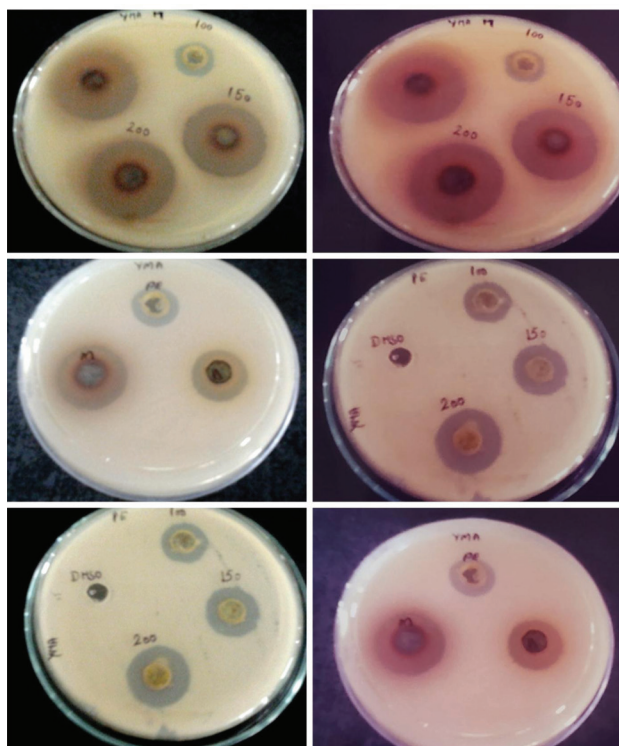


Figure 1: The results of evaluation of antibacterial activity.

Phenolic constituents act as a trademark for malignant growth anticipation and are abundant in plant drugs (Pourreza, 2013; Thaipong, K., U. Boonprakob, K. Crosby, 2006). Flavonoids, along with other phenolic blends depict diverse pharmacological activities, such as anti-atherosclerotic, antitumor, antiviral, antifungal, antimicrobial, cardioprotective action, etc. (Tapas, Sakarkar, & Kakde, 2006) 1992. It has also been observed that free radicals possess a role in pathology of diabetes (Oberley, L.W. Olamide, A.A., Olayemi, O.O., & Demetrius, 2011). Extensive research has been conducted on polyphenols to conclude their antidiabetic activity (Mohan & Nandhakumar, 2014).

Conclusion

The conclusion of the conducted research can be summarized based on the previously mentioned results that the ethyl acetate fraction/ extract from Rajasthan of *Citrullus* Species i.e. (*C. Colocynthis* & *C. Lanatus*) fruits exhibit strong antimicrobial activity followed by the samples from Punjab and Haryana. It can be also stipulated that tannins, flavonoids & quercetin might be responsible for the documented in-vitro antimicrobial activity of these plants.

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Authorship Contribution

HS: Methodology, Investigation, Writing-Original draft, Writing-Review & Editing, HMM: Formal Analysis, Supervision, Project Administration.

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Conflict of Interest

There is no conflict of interest.

Declarations

It is an original data and has neither been sent elsewhere nor published anywhere.

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