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Potential wound healing activity of bornane dominating fraction extracted from *Aspergillus terreus*

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ABSTRACT
A bioactive fraction was isolated from the intracellular pigment of Aspergillus terreus isolated from the sediments of Banasura Sagar Dam, Wayanad. It was initially characterized using TLC, HPTLC, UV visible spectrophotometry, GC MS and GC MSMS. The further characterization of the fraction 9(1) using GC-MSMS chromatography showed the presence of a major compound , Bornane,2,2,5-endo,6-exo,8,9,10- heptachloro with an area % of 40.4 and molecular weight of 376. The compound was which was also detected during the GC-MS analysis of the crude pigment as 2,3-Norbornanedione, 1-chloro with an area normalization of 4.11%. Appreciable activity of the pigment in wound healing was demonstrated by closure of the wounds in adult wistar rats within 10 days of application of the crude pigment sample and 8 days for fraction 9(1) compared to 14 days for boric acid. The histopathological analysis revealed the migration of inflammatory cells, fibroblasts, revascularization at the site of injury with a healthy cellular texture and thick cell margins. One way ANOVA of the data indicates a significance level of 0.0318 for 1% crude pigment sample and 0.0247 for 0.1% fraction 9(1) to that of the set level of significance at 0.05.
Key words: *Aspergillus terreus, Bornane, Excision wound healing, Pigment*

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INTRODUCTION

The demand for the natural source of pigments is increasing day by day because of the positive health benefits of natural compounds. Various pigments are reported to be used in industries selling foodstuffs, cosmetics and pharmaceuticals [1]. Biologically active chemical compounds including antimicrobials, fungal toxins, immuno-modulatory agents and hypolipidemic agents are reported to be produced from *Aspergillus* [2].Various secondary metabolic products like terreineol, terreulactone A, terrain, terreic acid and aspulvinones reported to be isolated from *Aspergillus terreus*.[3],[4],[5],[6],[7].

A wound is a result of any physical injury that results in breakage or opening of the skin. There are different tissues and cell lineages participating in the healing process which includes cytokines, growth factors, their mediators, and the extracellular matrix proteins. The process of contraction of the wound involves blood clotting and aggregation of platelets, angiogenesis, build-up of fibrin, inflammatory responses and change or alteration in dermal structure and re-establishment of epithelial cel.ls and finally they are knit by collagen [8].

Bornane is a crystalline saturated terpene, C10 H18 which is regarded as the parent compound of borneol, camphor and related compounds such as 1, 7, 7–trimethyilhorbornane – called camphene. Due to the irritant, anti-bacterial and anti-fungal property, tri-terpenoids promote the process of wound healing resulting in contraction of wounds and elevates the epithelialisation rate [9], [10], [11]. The medicinal property of plants typically in the process of wound healing lies in their capability of producing bioactive phytochemical secondary metabolites like alkaloids, saponins, tannins, flavonoids, essential oils, phenolics and terpenoids. These compounds produce definite physiological actions on the human body [12]. The microbial cells are also known to produce various secondary metabolites and hence they

contribute to many different biological properties. The *in vitro* antibacterial, anthelmintic, anti oxidant and cytotoxic activity of the intracellular pigment extracted from *Aspergillus terreus* has been reported [13], [14].

In the current paper we are discussing the potential wound healing activity of a terpenoid compound, Bornane extracted from the pigment extract of *Aspergillus terreus.*

MATERIAL AND METHODS

Sample collection

The soil sample for the isolation of *Aspergillus terreus* was collected from the sediments of Banasura Sagar Dam, located at Wayanad, Kerala.

Isolation and characterization of the organism

A red pigmented fungal culture was isolated on Starch Casein Nitrate (SCN) agar medium. The organism was identified by macroscopic, microscopic and molecular characterization by sequencing 5.8s rRNA at IMTECH, Chandigarh. The organism was studied for its pigmentation on various other fungal growth medium like Potato Dextrose Agar (PDA), Saborauds Dextrose Agar (SDA) and Malt Extract Agar (MA).

Extraction and production of pigment

The solvent extraction method using ethyl acetate was used for extracting the intracellular pigment produced by the organism. Mass cultivation using submerged fermentation was done employing SCN broth medium and was subject to incubation for 7 days at 30°C.

Purification of the crude pigment

Silica gel column chromatography

The partial purification of the crude pigment was done by column chromatography using silica gel. The solvent system used was hexane and ethyl acetate, in increasing order of polarity. It was performed by stuffing a chromatography coloumn with silica gel dissolved in hexane. The column was developed initially with hexane, a low-polarity solvent and the polarity was increased gradually using ethyl acetate until the entire fractions of the pigment was eluted.

Characterization of crude pigment and fraction 9(1): TLC, HPTLC and UV Visible spectrophotometry, GC MS and GC MSMS

TLC and HPTLC (Camag, Switzerland) of the pigment (crude and fraction 9(1) was performed using hexane: ethylacetate solvent system in the ratio of (9:1). The UV Visible spectrophotometry was done in the entire scan range of 1100-199 nm using UV visible spectrophotometer 1800, Schimadzu made in Japan. A single quadruple GC MS (GCMS-QP2010 Ultra, Shimadzu, Japan) and a triple quadraple GC MSMS (Thermofisher scientific) were performed to analyze the dominant compounds in the fractions of the pigment after chromatography.

Invivo wound healing studies

In the excision wound healing activity of the pigment [crude and fraction 9(1)], adult wistar rats of either sex weighing approximately 200- 250 gm were used. Six animals each were divided into three group's namely positive, negative and treatment groups. The pigment was dissolved in white paraffin base. The positive group was treated with boric acid; the negative group with the vehicle, white paraffin base and the treatment group with the pigment extract. The rats were given thiopentone anesthesia. The mice were prepared by depilating its back hairs and on the dorsal inter- scapular region, a circular wound was created by excising the skin with approximately 500 mm^2 area and the wounds were left open and surface sterilized [15]. The positive control, negative control and the pigment extracts (crude and partially purified fractions) were applied topically once a day till the wound was completely healed. The closure of wounds was monitored by tracing it in a butter paper and then transferring to graph sheets. The percentage wound healing process was expressed as:

% wound healing= {wound area on day n/ wound area on day 0} x 100.

The excision wound healing study was done for the crude pigment extract formulated as 1% ointment in white paraffin base as well as for the purified fraction 9, formulated as 0.1% ointment in white paraffin base.

Histopathology

The tissue sample for histopathological analysis was surgically removed from the healed skin of each group of mice [16]. The tissue samples were fixed using 10% formalin and were embedded in paraffin wax. The tissues were cut into serial sections of 5µm thickness and were embedded with paraffin wax. They were stained using stains such as eosin and haematoxylin. The samples were examined qualitatively for various pathological features including necrosis, ulceration, congestion, vascularization, epithelialization, polymorphonuclear leukocytes (PNL), mononuclear cells, fibroblasts and edema.

Statistical analysis

The percentage wound healing was statistically analysed using one way ANOVA. The statistical significance of the values of 'p' were set at, $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation and characterization of the organism

The macroscopic appearance of the red pigmented fungus isolated on SCN media showed fluffy, white and cottony spores with reverse side intracellular red pigmentation (Picture 1). Microscopically, the organism was found to possess septate hyphae with numerous phialide type conidia holding unbranched straight conidiophores. The top of the conidiophores bulged to become vesicles with phialide and metulae. The partial sequencing of 5.8s r RNA confirmed the organism as *Aspergillus terreus*. The production of the pigment was observed only on SCN medium which may be attributed to the neutral pH or the presence of NaCl and other minerals in the medium.

An Italian priest and biologist Pier Antonio Micheli first catalogued *Aspergillus* in 1729 [17]. Based on the morphological, physiological and phylogenetic characters, *Aspergillus* comprises a broad, diverse group of organisms. Currently, *Aspergillus* represents an asexual spore-forming structure common to all *Aspergillus* species and around one third of these species are recognized to have a sexual stage. They have a significant impact on food production, biotechnology, indoor environments and human health. The genus *Aspergillus* is diversified due to their high economic and social impact. *Aspergillus* is defined as a group of fungi which exist in its asexual state with the formation of conidia. They are ubiquitous and occur in various habitats. They are known food spoilers, produce mycotoxins and are the major contributors of human and animal infections.

Extraction and pigment production

The extraction of the intracellular pigment from *Aspergillus terreus* was done using ethyl acetate. After submerged fermentation, the mycelia was revived by filtration and then extracted. It was subject to rota vacuum evaporation and stored for further applications.

The exclusion of pigments from cells can be done by a selection of various extraction schemes. The methods used are experiential and was done by extraction with polar or non-polar single solvent or by solvent mixtures [18], [19]. The extraction of the intracellular pigment from *Streptomycetes sp.,* was done using ethyl acetate and the evaporated extract was used for testing the antioxidant and antimicrobial activity against biofilm forming bacteria [20],[21], described a method for extraction of the red pigment from *Serratia marcesens* using 5% acetone. The methods adopted to eliminate pigments from whole cells may depend upon various properties like solubility, differences in location and/or binding of pigments [22], described the segregation of a pigment-glycoprotein compound from *Sarcinaflava* (*Micrococcus luteus)* in which the pigment remains covalently attached to the composite through the sugar moiety.

Purification of the crude pigment

The crude pigment was subjected to partial purification using silica gel column with the eluents (hexane: ethyl acetate) in the increasing order of polarity (Picture 2). The fractions were collected into clean dry test tubes and were serially numbered from1 to 105. The fractions were evaporated using rota vacuum evaporator and screened using TLC for the presence of single compounds detectable in the fluorescent region. The selected compounds were assayed for the various biological activities. The most bioactive fraction, 9 (1) was taken for further characterization and application studies.

Characterization of the pigment: Thin layer chromatography, HPTLC, UV Visible spectrophotometry, GC MS and GC MSMS

The thin layer chromatogram of the crude pigment revealed three distinct spots in the fluorescent region at 366 nm with *Rf* of 0.05, 0.59 and 0.81. The thin layer chromatogram of the partially purified pigment showed a single compound with an *Rf* of 0.61in the fluorescent range.

The HPTLC of the crude pigment revealed the presence of at least 12 different polyvalent compounds in the fluorescent range. The maximum peak area of 32.62% was obtained for compound 2. The HPTLC of sample 9 (1) showed the presence of a single compound with start point *Rf* of 0.38 and end point *Rf* of 0.55 with peak area of 705.2 AU and an area % of 41.25.

The UV- Visible spectrum of the crude pigment showed multiple absorption peaks between 200 and 400 nm. Absorption of 3.157, 3.967 and 3.189 was observed at 224, 264 and 352 nm respectively. The UV-Visible spectrum of the partially purified 9(1) sample showed an absorption peak of 4.00 at 276 nm.

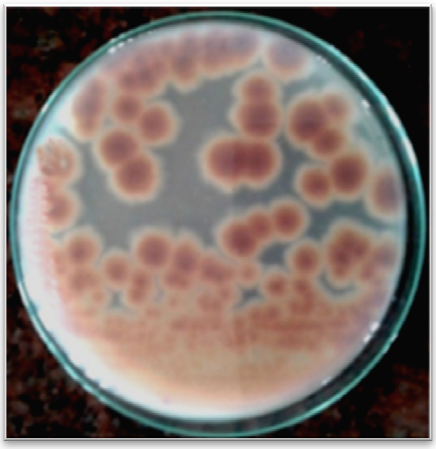
The results of further characterization of the fraction using GC-MSMS chromatography showed the presence of a major compound with an area % of 40.4 and molecular weight of 376. The compound was Bornane,2,2,5-endo,6-exo,8,9,10- heptachloro which was also detected during the GC-MS analysis of the crude pigment as 2,3-Norbornanedione, 1-chloro with an area normalization of 4.11%.

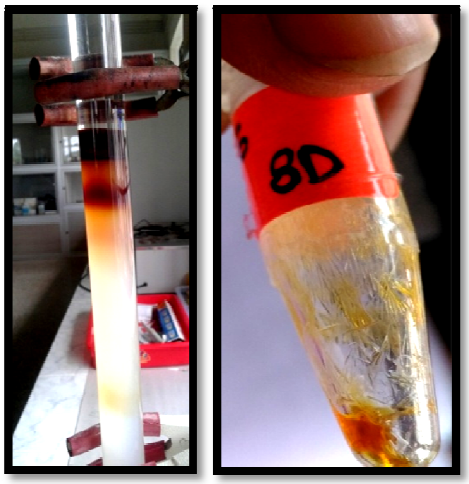
***In vivo* wound healing activity**

The excision wound healing study was done for the crude pigment extract (1% ointment) and for the partially purified fraction 9(1) (0.1% ointment) (Figure 1 and 2). Appreciable activity was demonstrated by the closure of wounds within 10 days of application of the crude pigment sample and 8 days for fraction 9(1) compared to 14 days for boric acid (Picture: 3). The histopathological analysis revealed the migration of inflammatory cells, fibroblasts, revascularization at the site of injury with a healthy cellular texture and thick cell margins (Picture: 4). One way ANOVA of the data indicates a significance level of 0.0318 for 1% crude pigment sample and 0.0247 for 0.1% fraction 9(1) to that of the set level of significance at 0.05.

Many studies have been performed on the evaluation of wound healing activity of plants. Natural wound healing agents are always a choice drug when compared to synthetic ones due to its contra-indications. The wound healing process normally takes over a course of more than 21 days without treatment, if it is not infected by bacterial agents and provided the immune system of the host remains good. The treatment with the standard drug used in the study, boric acid takes approximately 14 days for the scab formation. In the present study, the pigment has shown to have a potent wound healing activity, with the crude and purified fraction of the pigment with epithelialization periods of 10 and 8 days respectively. The property can very much be related to the activity of terpenoid compounds like Bornane, 2, 2, 5- endo, 6-exo, 8, 9, 10- heptachloro derivatives of chlorobornanes. Lupeol, a tri-terpenoid compound has been reported to be one of the active wound healing substances which mediate the cutaneous wound healing better than the reference drug nitrofurazone [23]. It is known that a compound with potential antioxidant activity fastens up the process of wound healing due to the generation of free radicals. Hence the appreciable antioxidant activity of the pigment already reported [14] may significantly contribute to the wound healing potential by the chlorobornane fraction.

Picture 1: Macroscopic appearance of *Aspergillus terreus*





Picture 2: Purification of the crude sample [Fraction 9(1)]

Figure 1: %Wound area of 1% crude pigment sample

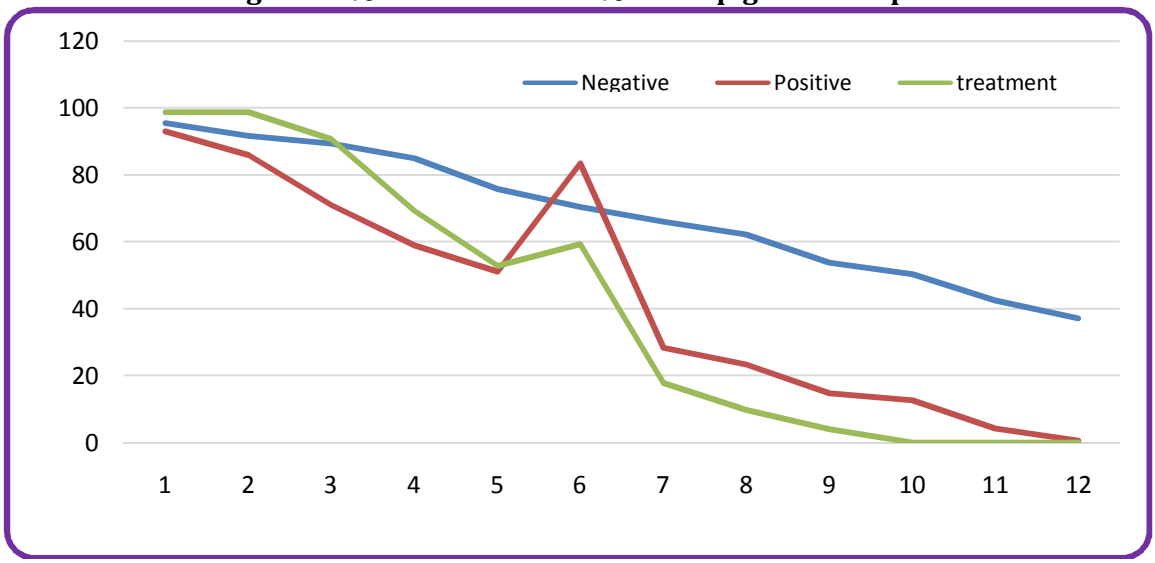
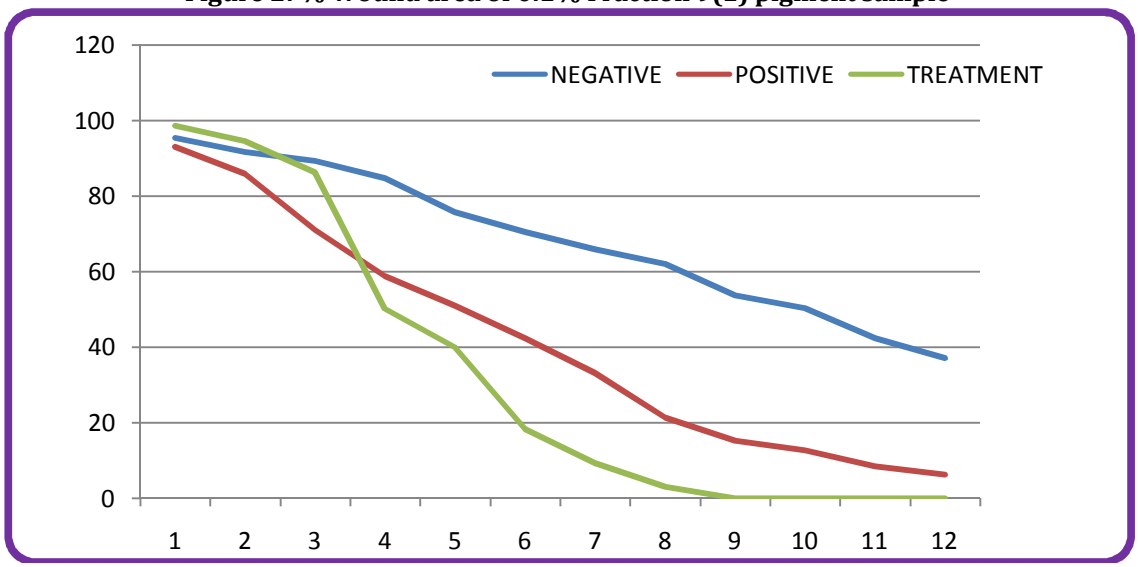
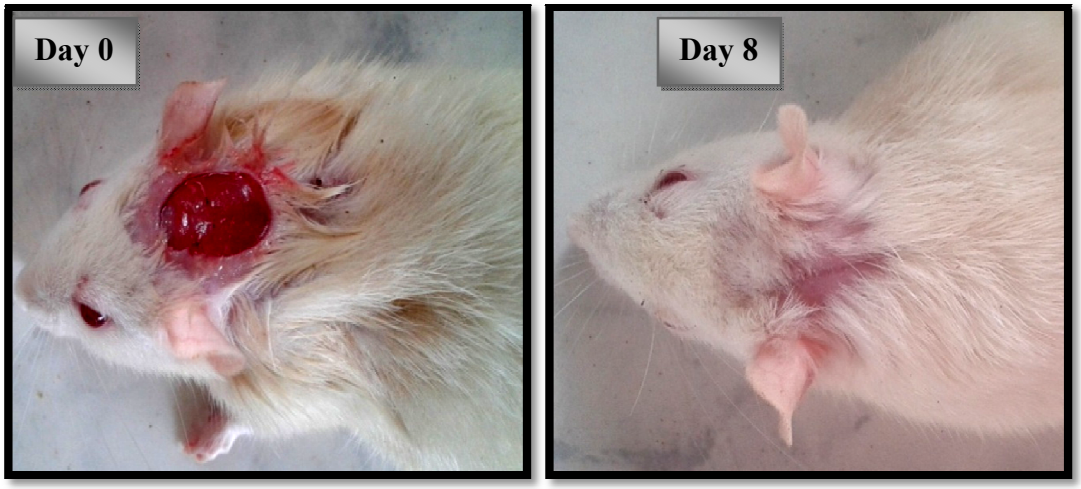


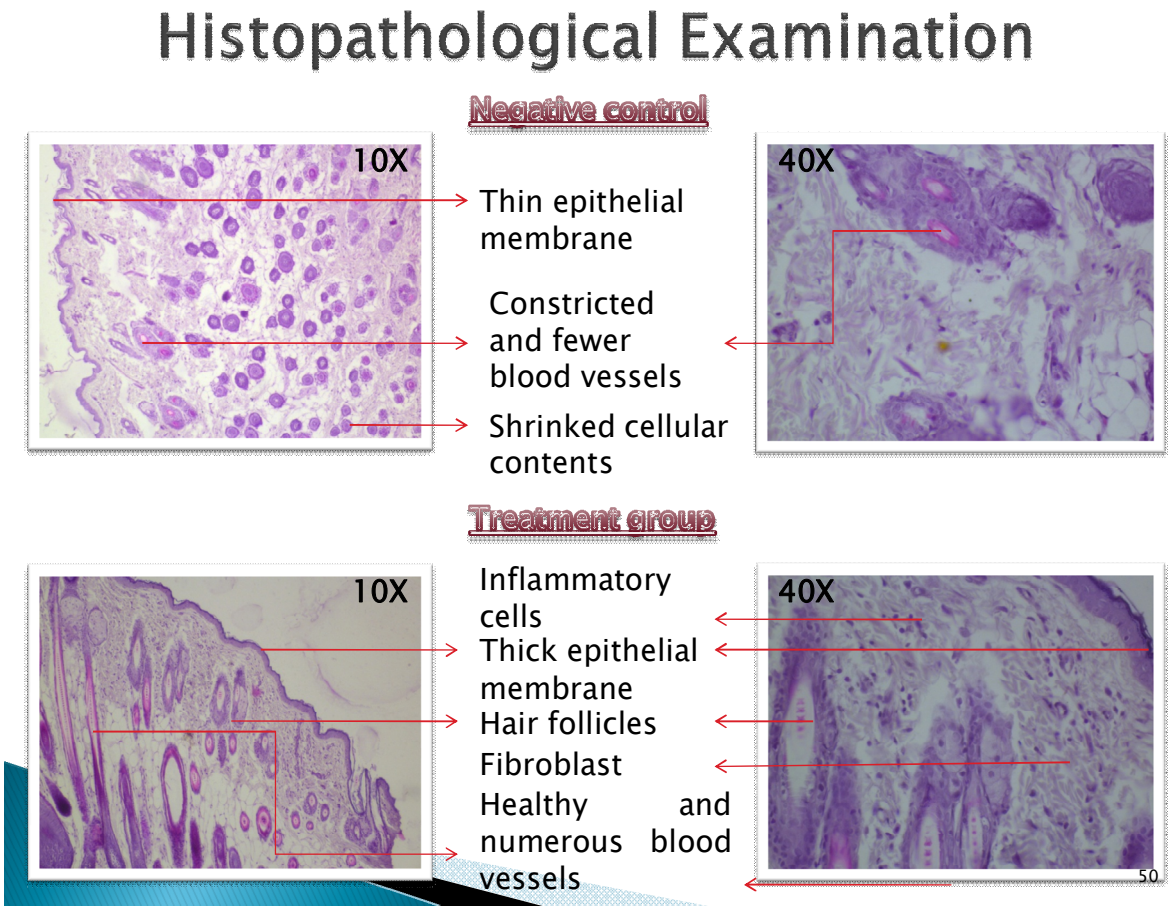
Figure 2: % Wound area of 0.1% Fraction 9(1) pigment sample



Picture 3: Wound healing of the fraction 9(1)



Picture 4: Histopathological Examination



CONCLUSION

The present study focuses on fraction 9(1) of the intracellular red pigment extracted from *Aspergillus terreus*, which shows dominance of Bornane as the major compound. The compound is shown to possess potent wound healing activity on adult wistar rats. The future work is aimed at purification, characterization and analyzing the wound healing potential of the purified individual compound, Bornane.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

ETHICAL STATEMENT

The present study had approval from the institutional Ethics Committee (IAEC/CVASMY 2/16-17, 22 December 2016.

FUNDING

None

AUTHORS CONTRIBUTION

All authors have made substantial direct and intellectual contribution to the work and approved it for publication.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

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COMPETING INTEREST
The authors have declared that no competing interest exists.

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