Asian Journal of Medical Sciences 2(4): 195-200, 2010
ISSN: 2040-8773
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Submitted Date: July 03, 2010 Accepted Date: August 29, 2010 Published Date: August 30, 2010

Antagonistic Potentials of Marine Sponge Associated Fungi *Aspergillus clavatus* MFD15

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Abstract: The development of resistance to multiple drugs is a major problem in the treatment of these infectious diseases. Multidrug Resistant *Staphylococcus aureus* (MRSA) and *Candida* sp, the major infectious agents have been recently reported in quite a large number of studies. With more intensive studies for natural therapies, marine-derived products have been a promising source for the discovery of novel bioactive compounds. A total of 45 marine fungi were isolated from the two sponges *F. cavernosa* and *D. nigra* were screened for antimicrobial activity against pathogenic bacteria and fungi. The novel basal media formulated in the present study resulted in increased frequency of fungal isolates when compared to all other media used in the present study. The cell free supernatant of fungi exhibiting the broad spectrum of activity was subjected to chemical analysis using different chromatographic systems including TLC, Column and GC-MS. Of the 15 fungal strains, 20% (3 strains) showed potential antagonistic activity against a panel of clinical pathogens used in the present study. Based on the antimicrobial activity of the isolates, *Aspergillus clavatus* MFD15 was recorded as potent producer displaying 100% activity against the tested pathogenic organisms. The TLC of the crude ethyl acetate extract produced 3 spots with Rf values of 0.20, 0.79 and 0.95, respectively. The active TLC fraction was purified in column chromatography which yielded 50 fractions. The active column fractions were combined and analyzed with FT-IR, UV-Vis and GC-MS. The chemical analysis of the active compound envisaged the active compound to be a triazole, 1H-1,2,4 Triazole 3- carboxaldehyde 5- methyl. The triazolic compound was bacteriostic for *S. aureus* and bactericidal for *E. coli.* The triazole treated fabric showed 50% reduction in the growth of *E. coli, S. aureus,* and *S. epidermidis.* Thus the purified compound can find a place in the database for the development of fabrics with antimicrobial properties. This is the first report that envisaged the production of triazole antimicrobial compound from sponge associated marine fungi from the Indian coast.

Key words: Antimicrobial compound, *Aspergillus clavatus*, marine fungi, marine sponge, multidrug resistant pathogens

INTRODUCTION

Microbial infections of the skin and underlying tissues are among the most frequent conditions encountered in acute ambulatory care (Fung *et al.,* 2003; Naimi *et al*., 2003). Skin infections (such as cellulites, erysipelas, trauma and wound related infections) especially when associated with co-morbid conditions and/or bacteraemia, may lead to severe complications and hospital admission. In some cases they can be the cause of extensive morbidity and mortalility (Fung *et al.,* 2003). Such dermal infectious diseases are leading health problems with high morbidity and mortality in developing countries. The development of resistance to multiple drugs is a major problem in the treatment of these infectious diseases. Multidrug resistant *Staphylococcus aureus* (MRSA) and *Candida* sp., the major infectious agents have been recently reported in quite a large number of studies. The increased prevalence of antibiotic resistant bacteria due to the extensive use of antibiotics may render the current antimicrobial agents ineffective in the near future (Tanaka *et al.,* 2006). This lacuna warranted the need of new bioactive compounds to emerging and reemerging infectious diseases.

Textile are known to be susceptible to microbial infection, as textiles provide large surface area and absorbs moisture which is required for microbial growth (Cardamone, 2002). Thus microbial attack of textile often leads to objectionable odor, dermal infection, allergic responses and other related diseases (Thiry, 2001). Thus it necessitates the development of clothing with antimicrobial properties to combat skin infections. The rate of discovery of novel compounds from terrestrial sources has decreased whereas the rate of re-isolation of known compounds has increased dramatically. Thus it is crucial to explore novel organisms from pristine habitat as

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sources of novel bioactive secondary metabolites. Marine organisms hold a position in the database for the investigation of numerous natural products. As marine conditions are extremely different from their terrestrial counterparts, it is surmised that marine organisms have different characteristics from those of terrestrial ones and therefore might produce different types of bioactive compounds.

Although the first potential antibiotic was obtained from a fungi *Penicillium*, less studies are being carried out in order to isolate and purify antibiotics from fungi. Thus in the present study an attempt was made to isolate fungi from the marine sponges and to screen the antimicrobial triazole producer. Triazoles are most widely used antifungal agents that offer activity against many fungal pathogens without the serious nephrotoxic effects. These triazoles are commonly synthetic and find wide application as topicals and in agriculture. But surprisingly in the present study a triazole have been purified from the fungal strain *A. clavatus* MFD15 and the purified triazole showed bioactivity against multiresistant bacteria and *Candida albicans*. Thus with the view point of prevention of dermal infections, the purified triazole was analysed for antimicrobial activity. To our knowledge this is the first study where fungal triazoles were purified and evaluated for the treatment of fabrics, to develop clothing with antimicrobial activity.

MATERIALS AND METHODS

Collection of sponges: The present study was carried out in Marine Bio-prospecting Laboratory, Bharathidasan University, Trichy, India between January and December 2009. Two marine sponges, *Fasciospongia cavernosa* and *Dendrilla nigra* were collected off (January, 2009) from the southwest coast of India (Vizhijam coast, Thiruvananthapuram, Kerala) by SCUBA diving at 10-12 m depth. Samples were surface cleaned with aged sterile seawater and sterilized with 70% alcohol. Then the samples were kept in sterile incubator oven for 1 h at 40ºC to dry the surface and transported to the laboratory and frozen at -20ºC immediately in sterile ziplap covers. Voucher specimens, photographs and videographs of the specimens and habitat were retained in the Marine Bio-prospecting laboratory, Bharathidasan University, Trichy, India for future reference.

Isolation of fungi: A measured area of sponge tissue ($1 cm^3$) was excised from the middle internal mesohyl area of the sponge using a sterile scalpel. The excised tissue was then homogenized with sterile aged seawater, using a sterile mortar and pestle. The resultant homogenate was serially diluted until 10^{-6} and preincubated at room temperature for 1 h for the activation of dormant cells.

Table 1: The basal media formulated for the selective isolation of marine fungi

Salt compositions (g/L)		
KH_2PO_4	-	0.2
NH_4Cl	-	0.25
KCl	-	0.5
$CaCl_2$	-	0.15
NaCl	-	1
$MgCl_2$	-	0.62
Na_2SO_4	-	2.84
HEPES	-	10mM (pH 6.8)
Yeast extract	-	0.05
Peptone	-	0.05
Dextrose	-	0.05
Aqueous sponge extract		100 mL
Trace element solution		1 mL
Vitamin solution		1 mlL

Table 2: Panel of multiresistant organisms used in the present study

Gram negative	Gram positive	Fungi
Klebisella pneumoniae P1	*Staphylococcus aureus* P4 (MRSA)	*Candida albicans*
Proteus mirabilis P2	*Staphylococcus epidermidis* P5	(*Fluconazole resistant*)
Salmonella typhi P3		

The aliquot was plated on various isolation media including a basal medium developed in the present study (Table 1) and other standard media such as marine agar, sabouraud dextrose agar, potato dextrose agar, starch casein agar, yeast malt extract agar, glycerol asparagine agar, and peptone yeast iron agar. The inoculated plates were incubated at room temperature (27±2ºC) for 14 days. Morphologically distinct fungal spores were isolated by successive subculturing on basal medium.

Screening for potential antagonistic producer: Determination of potential antagonistic fungi was performed by double layer agar diffusion method using the Cell Free Supernatant (CFS) collected from different fungal isolates grown by stationary culture on the basal medium. The CFS of the fungal isolates were tested against 6 pathogenic bacteria (2 g positive, 3 g negative) and *Candida albicans* (Table 2). The clinical pathogens were screened as multidrug resistant and deposited at Biomedical diagnostic Laboratory, Bharathidasan University. The test bacterial strains were maintained on nutrient agar slants at 4ºC. The *Candida albicans* was maintained on SDA slants at 4ºC.

Cultivation of *A. clavatus* MFD15: *A. clavatus* MFD15 was grown by surface culture in a 500 mL Erlenmeyer flask containing 200 mL of production medium (basal medium used in the present study). The flasks were incubated without shaking for 7 days at room temperature.

Extraction and purification of antimicrobial metabolites from *A. clavatus* MFD15: The CFS was separated by centrifugation at 8000xg for 15 min. The

CFS was acidified to pH 2.0 with 1 N HCl and further extracted twice with equal volume of ethyl acetate. The solvent phase was separated and concentrated in a rotary vacuum evaporator (Yamato). The residue obtained was dried in a vacuum dessicator and dissolved in ethyl acetate. The dried residue was checked for antimicrobial activity against a panel of microbes used in the present study.

The evaporated ethyl acetate extract was applied to the silica plates and placed in a chromatographic chamber saturated for minimum 3 h with mobile phase (chloroform: methanol, 10:1). After developing, the plates were dried for few hours at room temperature prior to spraying with ASE reagent (Anisaldehyde: sulphuric acid: ethanol, 1:1:9). The plates were subsequently heated at 110ºC for 2 min and spot appearance, location and color were analyzed (Vanderhaeghe and Kerremans, 1980). The *Rf* values were determined manually.

The spots were scrapped out and checked for bioactivity. The active fractions were evaporated to dryness and loaded onto silica gel column previously equilibrated with hexane: ethyl acetate (1:1). The fractions were separated by isocratic run with 1% acetic acid in water/CH_3CN 55:45; flow rate 20 ml/min. In total 50 fractions were collected and analyzed for bioactivity. Fractions 41-45 were then combined and subjected for chemical analysis.

Identification of the antimicrobial metabolite: The UV spectrum of the active column fraction was recorded on Shimadzu IR-470 model. The spectrum was scanned on 200 to 400 per cm range. The infrared spectrum of the active column fraction was recorded on Shimadzu IR-470 model. The spectrum was scanned on 400 to 4000 per cm range. The spectra were obtained using potassium bromide pellet technique. Potassium bromide (AR grade) was dried under vacuum at 100ºC for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra were plotted as intensity versus wave number. Then the active ethyl acetate extract was subjected to gas chromatography-mass spectrometry (GCMS) analysis. An Agilent GC-MS system equipped with a fused silica capillary tube was used to analyze the components in this active fraction. The data was processed by GC-MSD Chemstation column condition was programmed as column oven temperature 150ºC (4 min)-4ºC/min, temperature of inject port 250ºC and detector port 280ºC (Roy *et al.*, 2006).The peaks of the gas chromatography were subjected to mass-spectral analysis. The spectra were analyzed from the available library data. NIST MS search (version 2.0; included with NIST'02 mass spectral library, Agilent p/n G1033A)

Determination of minimal inhibitory and minimal bactericidal concentration: The MIC was determined by broth dilution method. The multidrug resistant *E. coli, S. aureus* and *Candida albicans* were used for determination of MIC and MBC. The 96 well microtitre plates were filled with 0.1 mL of varying concentration of active fractions prepared in Muller Hinton broth with culture was added to it. The microtitre plates were incubated at 37ºC for 18 h. One row served as positive control (antibiotics) and one as negative control (ethyl acetate). After incubation, the OD was read at 610 nm in an ELISA reader. For measuring MBC, irrespectively all the MIC cultures were plated on Mulleer Hinton Agar and incubated at 37ºC for 24 h. A reduction in the number of viable colonies compared with the culture of the initial inoculum was noted. The ratio of MBC/MIC was calculated as an index of bacetriostatic and bacetricidal.

Antimicrobial activity of the treated fabric with triazole fraction: Cotton fabric was purchased and washed with non-ionic detergent at 95ºC for 4 h. The fabric was washed thoroughly with water; air dried at room temperature and soaked in the purified triazole fraction for 2 h. The solvent was evaporated to dryness and antimicrobial activity of the soaked fabric was analysed. The reduction of bacterial growth by the purified compound was determined by measuring the absorbance at 610 nm. The reduction in microbial growth was calculated using the formula RZ = (BZ-AZ)/AZ x 100, where RZ represents the percentage reduction in bacterial growth, BZ represents the absorbance of the sample with test microbe and untreated fabric, AZ indicates the absorbance of the sample with test microbe and treated fabric (Rajni *et al.,* 2005).

RESULTS AND DISCUSSION

Isolation and identification of endosymbiotic fungal community: A total of 45 marine fungi were isolated from the two sponges *F. cavernosa* and *D. nigra*. Based on the stability of subculturing, 15 marine fungi were obtained and deposited at the Marine Bioprospecting Laboratory, Bharathidasan University under the Marine Fungal Depository (MFD) series (MFD01-MFD15). In comparison with all other standard media used in the present study, the basal medium supplemented with high vitamin content significantly ($p<0.001$) increased the cultivation potential of sponge associated fungi (Fig. 1). The increased frequency of isolation was due to the presence of vitamins that have favored more of fungal species bring into culture. The envisaged results have been supported by earlier reports where thiamaine and pyridoxine supplemented media enhanced the growth of *Cladosporium phlei*, *Rhizoctonia zeae*, *R. oryzae* and *Waitea circinata*. The basal medium not supplemented with vitamins caused dramatic pH lowering after a long period of incubation. As the sponge associated fungi are

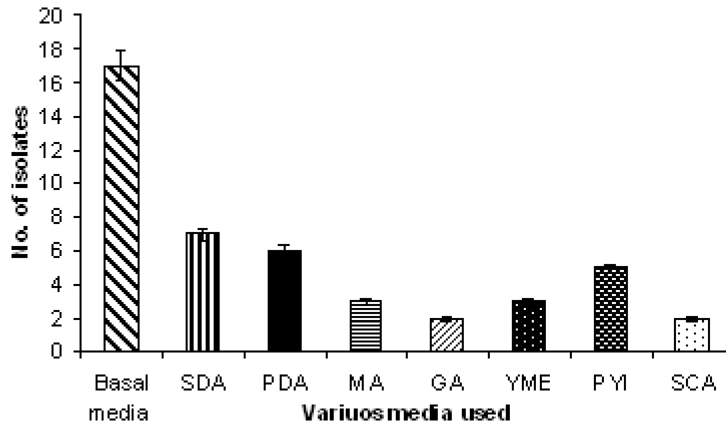

Fig. 1: The number of isolates obtained on various standard isolation agar in comparison with the basal media formulated in the study. The number represents the number of fungal isolates selected depending on the difference in their characteristics on the master plate and microscopic observation. SDA- sabouraud dextrose agar; PDA- potato dextrose agar; MA- marine agar; GA- Glycerol asparagine agar; YME- Yeast malt extract agar; PYI- peptone yeast iron agar; SCA- Starch casein agar

Table 3: Number and taxonomic identification of sponge associated marine fungi

Taxon	No. of isolates	Relative frequency (%) [a]
Ascomycte sp.	10	67
Chaetomium sp.	2	13
Cladosporium sp.	2	13
Colletotrichum sp.	1	6

[a]: Relative frequency of isolation used for indicating species abundance was calculated as the number of isolates of a taxon divided by the total number of stable isolates (15) from the two marine sponges

from a marine environment, they required an alkaline pH for their growth. Thus a lowered pH provides an insalubrious environment for the fungal isolates to grow. Ascomycete was found to be the predominant taxon with 67% of relative frequency of isolation followed by Chaetomium, Cladosporium and Colletotrichum taxons (Table 3).

Antimicrobial activity of the fungal isolates: Of the 15 fungal strains, 20% (3 strains) showed potential antagonistic activity against a panel of clinical pathogens used in the present study (Table 4). Among these 3 isolates MFD15 needs special mention as it exhibited antimicrobial activity against all pathogenic test strains. The CFS of MFD15 showed almost similar activity against gram positive, gram negative bacteria and *Candida* sp. The antibiogram was normally distributed between gram positive and negative bacteria. It has been

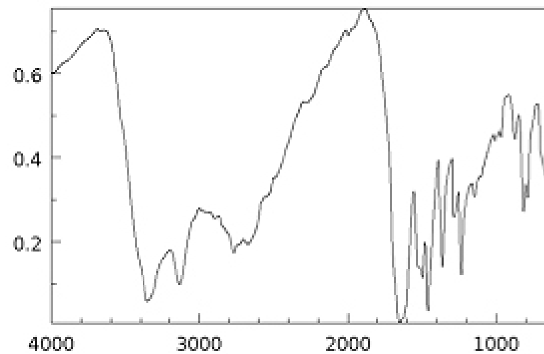

Fig. 2: FTIR spectrum of the active column fraction

reported that the extraction method had definite effect on the isolation of bioactive principles (Selvin *et al.*, 2009). But such definite effect was not observed in the present study envisaging that MFD15 CFS contain active compound that are extractable with ethyl acetate and can exhibit similar antimicrobial activity against a wide range of pathogens irrespective of their types.

Extraction and purification of antimicrobial compound: It has been predicted that MFD15 produce substances belonging to triazoles based on the TLC analysis. TLC analysis of the ethyl acetate extract

Table 4: Antimicrobial activity of the fungal isolates

S/P	MFD1	MFD2	MFD3	MFD4	MFD5	MFD6	MFD7	MFD8	MFD9	MFD10	MFD11	MFD12	MFD13	MFD14	MFD15
P 1	0	530.66	0	615.44	907.46	0	379.94	452.16	254.34	78.5	113.04	314	206.5	452.16	907.46
P 2	0	530.66	0	314	615.44	0	379.94	379.94	200.96	113.04	113.04	200.96	379.94	379.94	917.26
P 3	0	314	0	379.94	452.16	0	254.34	254.34	113.04	78.5	78.5	153.86	200.96	200.96	915.44
P 4	0	0	0	0	0	113.04	0	0	0	0	0	0	0	0	926
P 5	78.5	254.34	0	200.96	200.96	153.86	153.86	153.86	113.04	0	156.86	153.86	153.86	200.96	903.84
P 6	0	452.16	0	452.16	452.16	153.86	452.16	452.16	254.34	200.96	113.04	113.04	314	254.24	906.5

The values are presented as mean of triplicates performed as area of the inhibition zone in mm^2

P1: *Salmonella*, P2: *Proteus*, P3: *Klebsiella*, P4: *Staphylococcus aureus*, P5: *Staphylococcus epidermidis*, P6: *Candida*

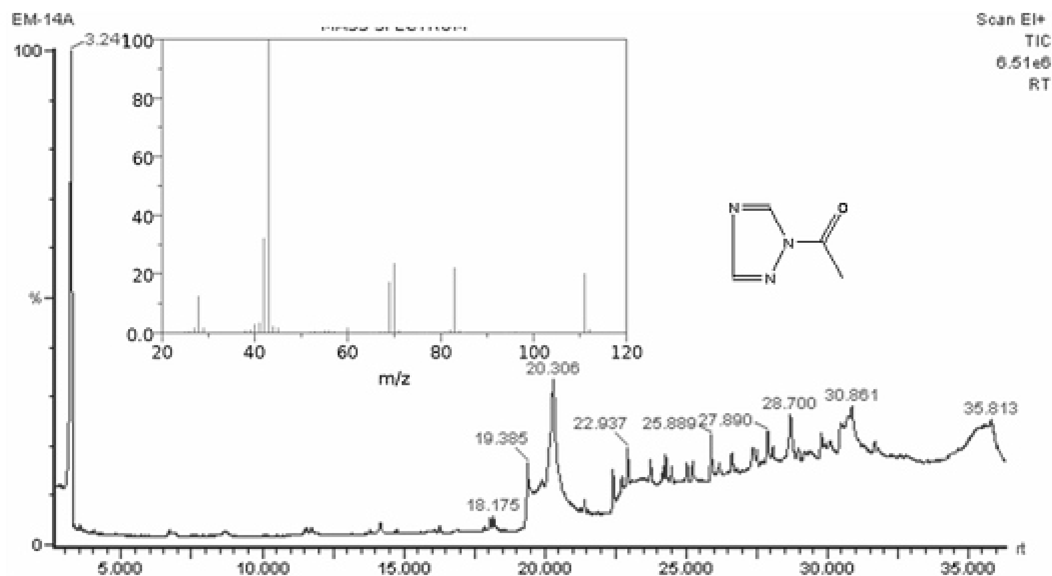

Fig. 3: GC-MS spectrum of the active fraction

Table 5: MIC and MBC values of the purified compound against the test organisms

Test organism	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC
E. coli	800±10	400±5.3	0.5
S. epidermidis	1600±6	6400±3	4.0
Candida albicans	400±7	200±6	0.5

The values are presented as mean±S.D. (n = 6)

indicated the presence of more than one compound, but the compounds with *Rf* value of 0.79 exhibited activity against the pathogenic microbes tested. The spray reagent used in the study showed a variety of colors after interacting with different compounds on the TLC plate.

Chemical analysis of the antimicrobial compound: The FTIR spectrum exhibited absorption bands at 3400, 3100, 1650, 1450, 1390, 1200 and 900 per cm (Fig. 2) indicating the presence of groups including OH, NH, amines, aromatic rings, CH, CO and alkenes respectively. The UV-VIS spectrum of the purified fraction was found to be a broad band with fine structures between 230-270 nm. Thus the absorption band is a B-band identifying the chromophores to be aromatic. Further the spectrum displayed maximum absorption at 285 nm showing the presence of carbonyl chromophore. The GC-MS analysis of the purified compound showed a single prominent peak (Fig. 3). Retention time and molecular weight of the relevant peak was 3.241 and 111, respectively. The key fragment of the metabolite was 40 in EI ionization mass spectroscopy spectra. The MS data matched perfectly with a compound of molecular formula $C_4H_5ON_3$ which corresponds to a triazole, 1H-1,2,4 Triazole 3-carboxaldehyde 5- methyl in the NIST library.

Mechanism of antibiosis: The active antimicrobial fraction of MFD15 was tested against *E. coli, S. aureus* and *C. albicans* in order to determine the MIC and MBC (Table 5). The antimicrobial triazole from *A. clavatus* MFD15 exhibited antimicrobial activity that was comparable with that of rifampicin and flucanozole (positive control) against bacteria and fungi, respectively. The purified triazole exhibited strong antibacterial activity against *E. coli* and *S. aureus* with an MIC at 800 and 1600 µg/mL, respectively. The rifampicin positive control co-assayed showed antibacterial activity against *E. coli* and *S. aureus* with MIC 1200 µg/mL which was comparable with that of the purified compound. The bioactive compound was found to be bactericidal (MBC/MIC$\le$2) for *E. coli* but it was bacetriostatic (MBC/MIC$\ge$2) for *S. aureus.*

The purified triazole exhibited strong anticandidal activity against *C. albicans* with an MIC at 400 µg/mL. The fluconazole (positive control) co-assayed showed anticandidal activity with MIC 1200 µg/mL which was significantly ($p<0.001$) different from that of the purified compound. Further the compound was also found to be fungicidal displaying a MBC/MIC $\le$2.

Antimicrobial activity of the compound treated fabric: Purified triazole treated fabric showed maximum reduction (50%) in the growth of *E.coli*, *Staphylococcus aureus* and *S. epidermidis*. It displayed moderate reduction of 25% in the growth of *Klebsiella*, *Proteus* and *Candida*. The difference in antimicrobial activity in agar medium and in case of treated fabric can be due to interaction between some functional groups and the fabric

resulting in reduced antimicrobial activity. Inorder to facilitate similar antimicrobial activity some adjuvants and linking agents can be used. This is because the crosslinking agents can hold some cationic sites in its structure and can prevent any modifications of the functional group thus retaining the original antimicrobial activity (El-Tahlawy *et al.*, 2005; Giri *et al.*, 2009; Lim and Hudson, 2004; Tomsic *et al.,* 2009). The most noticeable result is that the triazole treated fabric was effective against *S. aureus*, the most commonly elevated clinical pathogen as it is the major cause of cross infections in hospitals as well as in commercial and home laundry practices. Moreover it is the major cause for skin and tissue infections, septicemia, endocarditis and meningitis (Bhat *et al.*, 2005).

CONCLUSION

In the present study, we demonstrated the basal medium significantly increased the cultivation of sponge associated marine fungus. This is the first report that envisaged the production of triazole antimicrobial compound from sponge associated marine fungi. Thus the bioactivity of the fungal isolates opened up a novel doorstep for the development of fabric treated with antimicrobial triazole in order to develop clothing to treat skin infections. The compound can also be used for wound dressings to prevent wound infections.

ACKNOWLEDGMENT

The authors wish to acknowledge the Central Electrochemical Research Institute, Kariakudi for different chemical analysis. Authors thanks to the management of Maxwell Scientific Organization for financing the manuscript for publication.

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