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In vitro Antimicrobial Evaluation of Compound Derived from *Streptomyces* sp. TC1 against *Xanthomonas oryzae* pv. *oryzae*

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The antimicrobial principle from the culture filtrate of *Streptomyces* sp. TC1 against bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Xoo) was isolated by bioassay guided separation technique. The structure of the isolated compound has been identified as 6-hydroxy-7-methoxycoumarin using extensive spectroscopic studies. The isolated compound evaluated for its biological properties including antimicrobial, antioxidant, and protein binding activities. The compound produced an inhibition zone of 13-19 mm against various isolates of bacterial leaf blight pathogen in agar well diffusion method. The isolated compound possessed moderate antioxidant activity in the tested antioxidant assays. The docking studies of compound were carried out with specific proteins of interest which responsible for bacterial leaf blight disease in rice. Based on the binding energy and number of hydrogen bonds formed in protein docking studies, it is inferred that isolated compound exhibit antimicrobial activity against receptor protein 3S8M and 3H2G. These results indicate that the *Streptomyces* sp. TC1 can be utilized as a biocontrol organism for the management of bacterial leaf blight disease in rice.

Key words: *Streptomyces*, Antimicrobial, Antioxidant, Coumarin, Docking.

Actinomycetes are gram positive organisms known for producing several bioactive metabolites with diverse biological activities[1]. The genus *Streptomyces* constitutes around half of the total soil actinomycete population[2] and widely recognized as industrially important microorganisms because of their ability to produce novel secondary metabolites having utilization in agricultural, pharmaceutical and industrial applications[3]. The secondary metabolites from streptomycetes exhibited varied levels of antioxidant activities. The antioxidant metabolites like diphenazithionin[4], dihydroherbimycin A[5] and protocatechualdehyde[6] are produced by *Streptomyces* sp. and used for industrial applications. Microbial derived antibiotic metabolites were used for plant disease management and considered to be a better alternative or supplement for chemical control measures[7]. El-Abyad *et al.*,[8] reported that *Streptomyces* sp. are widely studied for their biocontrol potential against plant pathogens because of their antibiotics producing nature.

Rice is perhaps the most widely cultivated food crop world over, whose production is constrained by diseases of fungal, bacterial and viral origin. Bacterial blight (BB) of rice, caused by

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Xanthomonas oryzae pv. *oryzae* (*Xoo*) is one of the oldest known diseases and was first noticed by the farmers of Japan in 1884. Subsequently, its incidence has been reported from different parts of Asia, northern Australia, Africa and USA[9]. Crop losses of 10–20% in moderate conditions or severe losses of up to 50% in highly conducive conditions have been recorded in several Asian and Southeast Asian countries[10]. Antagonistic bacteria are considered ideal biological control agents for obvious reasons, like rapid growth, easy handling, and aggressive colonization of the rhizosphere[11]. Bacterial antagonists have been evaluated with various degrees of success for the suppression of rice diseases of fungal origin. Certain strains of *Bacillus* spp. and *Pseudomonas* spp., have been used as biocontrol agents to suppress rice bacterial blight[12].

The present study describes the isolation and structure elucidation of antimicrobial metabolite from the culture filtrate of *Streptomyces* sp. TC1 against Xoo. The biological activities including antimicrobial against various isolates of bacterial leaf blight pathogen, antioxidant, protein interactions and docking studies with Xoo specific protein were also addressed.

MATERIALS AND METHODS

Microbial culture

The *Streptomyces* sp. TC1 was grown in Ken knight agar slants for five days at 28ºC and maintained under refrigerated conditions. The test culture *Xanthomonas oryzae* pv. *oryzae* (Xoo) from various source of rice varieties *viz.*, TN1, TNRH180, CO47, CO50, ADT39, ADT43 were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India.

Chemicals

The chemicals used in the present study were of analytical reagent grade. It was purchased from Himedia, Sigma, Qualigens, and SD Fine Chem., India.

Fermentation and isolation of antimicrobial metabolite

A seed culture of *Streptomyces* sp. TC1 was prepared by inoculating a loop of biomass into a 200 ml Erlenmeyer flask containing 100 ml of Ken knight broth and then incubating at 28 ºC for 3 days. A 10 % level of this inoculum was transferred into 1000 ml of production medium contained in 3 l Erlenmeyer flasks (15 in number). The production medium having the composition of soluble starch 1.0%, casein 0.03%, KNO_3 0.2%, NaCl 0.2%, K_2HPO_4 0.2%, $CaCO_3$ 0.002%, $MgSO_4.7H_2O$ 0.005% and $Fe_3SO_4.7H_2O$ 0.001% with pH 8.0. The inoculated production flasks were incubated for 7 days at 28ºC.

The crude bioactive compound produced in liquid culture medium was extracted through shaking with equal volume of ethyl acetate in a separating funnel. The pH of the culture filtrate was adjusted to 3 using dilute HCl prior to extraction process. The ethyl acetate concentrate obtained from fermentation broth were used for purification purpose. Purification of active fraction was carried out by silica gel (60-120 mesh) column chromatography. The column was eluted with a gradient elution using a mixture of *n*-hexane-$CHCl_3$ (0-100%), which afforded several fractions that were pooled based on their analytical TLC results and their antimicrobial activity was analyzed against Xoo. The fractions obtained with the mixture of *n*-hexane-$CHCl_3$ (74:26 v/v) was subjected to preparative TLC (PTLC) using *n*-hexane-$CHCl_3$ (85:15 v/v) as the mobile phase in order to purify compound.

Structural elucidation of isolated compound

The isolated compound studied extensively using spectroscopic techniques. ^{1}H-NMR (Nuclear Magnetic Resonance spectroscopy) and ^{13}C-NMR were recorded at 400 and 100 MHz, respectively in $CDCl_3$ with TMS as an internal standard by a JNM-GSX 400 spectrometer (JEOL) at Indian Institute of Technology, Madras. The FT-IR (Fourier Transform Infrared spectroscopy) measurement of sample was performed using the Perkin Elmer 1600 FT-IR spectrophotometer in a diffuse reflectance mode at a resolution of 4 cm^{-1} in KBr pellets and the absorption frequencies are expressed in reciprocal centimeters (cm^{-1}). The FT-IR analysis was carried out at Department of Chemistry, Bharathiar University, Coimbatore. GC-MS (Gas chromatography-Mass spectrometry) analysis was carried out by using JEOL JMS600H, Indian Institute of Chemical Technology, Hyderabad.

Screening of antimicrobial activity

In vitro antimicrobial activity of the isolated compound from *Streptomyces* sp. TC 1

was studied against various isolates of Xoo strains by agar well diffusion method[13]. The compound was diluted in 100% ethanol at the concentration of 1 mg/ml. The antimicrobial activity was evaluated by loading 50 µl/well. The standard antibiotic tetracycline used as a positive control at the concentration of 1 mg/ml. The antimicrobial spectrum of the compound was determined in terms of zone of inhibition. Ethanol was used as negative control. The control zones were subtracted from the test zones and the results are arrived.

Antioxidant assays

The antioxidant capacities of compound were estimated according to the procedure described by Pulido *et al.*,[14]. FRAP reagent (900 µL), prepared freshly and incubated at 37 °C, was mixed with 90 µL of distilled water and 30 µL of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37 °C for 30 min in a water bath. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 20 mmol/L TPTZ solution in 40 mmol/L HCl, 2.5 mL of 20 mmol/L $FeCl_3.6H_2O$ and 25 mL of 0.3 mol/L acetate buffer (pH 3.6). At the end of incubation, the absorbance readings were taken immediately at 593 nm using a spectrophotometer. Methanolic solutions of known Fe (II) concentration, ranging from 100 to 2000 µmol/L, ($FeSO_4.7H_2O$) were used for the preparation of the calibration curve. The parameter Equivalent Concentration (EC) is defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mmol/L $FeSO_4 \cdot 7H_2O$ EC_1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of 1 mM/L concentration of Fe (II) solution, determined using the corresponding regression equation.

Metal chelating activity

The chelation of ferrous ion by compound was estimated by the method of Dinis *et al.*,[15]. The test samples and standard EDTA were added to a solution of 2 mmol/L $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mmol/L ferrozine (0.2 mL) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was measured spectrophotometrically at 562 nm against the blank. The chelating activity of the compound was evaluated using EDTA as standard and results were expressed as µg EDTA equivalents/mg compound.

$ABTS^{ï%+}$ radical scavenging activity

The total antioxidant potential was measured by bleaching of ABTS radical cations[16]. $ABTS^{ï%+}$ radical cations were prepared by incubation of 150 µM/l (50 ml) with 2 M/l potassium persulphate (1.25 ml) for 2 hrs at 50Ú˜C in phosphate buffer, pH 7.0 (0.02 M/l)[17]. To 996 µl of the $ABTS^{ï%+}$ radical cation, 4 µl of the test sample were added. The absorbance of the sample was measured after 15 minutes at 734 nm. Trolox was used as standard and the results were expressed as mg of Trolox equivalents.

Radical scavenging activity using $DPPH^{\cdot}$ method

The $DPPH^{ï%}$ radical scavenging activity of the compound isolated from *Streptomyces* sp. TC1 along with standards Rutin and BHT (Butylated Hydroxy Toluene) were measured according to the method of Blios[18]. This method is based on the reduction of the free radical $DPPH^{ï%}$ (2, 2-diphenyl-1-picrylhydrazyl). The reaction takes place when 1 ml of $DPPH^{ï%}$ (0.1 mM solution of $DPPH^{ï%}$ in methanol) was mixed with 3 ml of the test sample containing 20 µg of compound at room temperature. After a reaction time of 30 minutes, absorbance values at 517 nm were measured. The percent inhibition of the $DPPH^{ï%}$ radical by the samples was calculated according to the formula:

$$\text{Percentage (\%) inhibition} = [(Ac - As)/Ac] \times 100$$

Where,

Ac - absorbance of the control;

As - absorbance of the test sample.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the isolated compound was measured according to the method of Klein *et al*.,[19]. The test compound at a concentration of 20 µg were added with 1.0 mL of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1.0 mL of DMSO (0.85% DMSO (v/v) in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90 °C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1.0

mL of ice-cold TCA (17.5% w/v). Subsequently, 3.0 mL of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and make the volume up to 1 litre with distilled water) was added and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm. The percent inhibition of the hydroxyl radical by the samples was calculated according to the formula:

Percentage (%) inhibition = [(Ac- As) /Ac] x 100

Where,
Ac - absorbance of the control;
As - absorbance of the test sample.

Superoxide anion radical scavenging activity

The superoxide scavenging ability of the test samples were assessed by the method of Nishikimi *et al.*,[20] with slight modification. About 1 ml of nitro blue tetrazolium (NBT) solution (156 ¼M NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 ¼M in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of test sample were mixed. The reaction started by adding 100 ¼l of phenazine methosulphate (PMS) solution (60 ¼M PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank sample. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The capability of scavenging the superoxide radical was calculated using the following equation:

Scavenging effect % = [(Ab-As)/ Ac] x 100

Where,
Ab- Absorbance of the blank,
As - Absorbance of the test sample

Protein binding studies and Fluorescence quenching of BSA by compound

The excitation wavelength of BSA at 280 nm and the emission at 346 nm were monitored for the protein binding studies[21]. The excitation and emission slit widths and scan rates were maintained constant for all of the experiments. A stock solution of BSA was prepared in 50 mM phosphate buffer (pH = 7.2) and stored in the dark at 4 °C for further use. A concentrated stock solution of the compound was prepared for analysis. Titrations were manually done by using a micropipette for the addition of the compound. A solution of BSA (2 µM) was titrated with various concentration of the compound (0, 0.5, 1.0, 1.5 and 2.0 µM) and its fluorescence spectra were recorded in the range of 290-450 nm, upon excitation at 280 nm.

The observed change in shift is mainly due to the fact that the active site in protein is buried in a hydrophobic environment. This result suggested a definite interaction of the compound with the BSA protein. To study the quenching process further, fluorescence quenching data were analyzed with the Stern-Volmer equation and Scatchard equation. The quenching constant (K_q) can be calculated using the plot of I_0/I versus [Q]. If it is assumed that the binding of compounds with BSA occurs at equilibrium, the equilibrium binding constant can be analyzed according to the Scatchard equation: $\log [(I_0-I) / I] = \log K_{bin} + n \log [Q]$, where K_{bin} is the binding constant of the compound with BSA and n is the number of binding sites. From the plot of $\log (I_0-I)/ I$ versus log [Q], the number of binding sites (n) and the binding constant (K_{bin}) have been obtained.

Protein docking studies

In the field of molecular modeling, docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. The isolated bioactive metabolite along with control (Tetracycline) docked with protein which is responsible for leaf blight disease in rice and the binding energy of molecule was calculated. The bioinformatics software used for the docking study is "AutoDock"[22]. The target proteins used for docking studies are esterase LipA (PDB-3H2G) and FabV (PDB-3S8M). The best docking scores indicates the strong binding of the molecule with the enzyme. The work is carried out in Centre for Plant Molecular Biology and Biochemistry, Tamil Nadu Agricultural University, Coimbatore. The LipA protein is a rice cell wall degrading enzyme produce by *Xanthomonas oryzae*[23]. Whereas the FabV (PDB-3S8M) is the most recently discovered class of ENR; Enoyl-ACP reductase (ENR) catalyses the last reduction reaction in the fatty acid elongation cycle in bacteria and is a good antimicrobial target candidate[24].

RESULTS

In the present study the antimicrobial metabolite from the culture filtrate of *Streptomyces* sp. TC1 is isolated by bioassay guided separation technique. The isolated compound subjected to spectroscopic studies including 1D and 2D NMR, GC-MS, FT-IR and the structure of the compound is arrived as 6-hydroxy-7-methoxycoumarin.

Structural elucidation of isolated compound

The compound (Figure 1) showed a molecular ion at *m/z* 192.15, $[M]^+$ (calculated for $C_{10}H_8O_4$, 192.17) in its GC-MS, suggesting the molecular formula of $C_{10}H_8O_4$. The IR spectrum of compound showed intense absorption bands at 3368 cm^{-1} (OH), 1680 cm^{-1} (C=O), 1058 cm^{-1} (C-O), 1611 and 1474 cm^{-1} (phenyl group). The ^{1}H-NMR spectroscopic data of compound revealed the presence of hydroxyl group at d 6.15 (1H, s), and four aromatic protons, of which one pair of AB doublets at d 6.27 (1H, d, *J* = 10 Hz, H-3) and d 7.60 (1H, d, *J* = 10 Hz, H-4), two singlets at d 6.93 (1H, s, H-8) and d 6.85 (1H, s, H-5) and a methoxyl group at d 3.96 (3H, s) (Table 1). The ^{13}C-NMR (APT) spectrum disclosed the presence of 10 carbons

Table 1. Spectroscopic characterization of isolated compound

^{1}H NMR	^{13}C (HSQC)	HMBC
δ 7.60 (1H, d, *J* = 9.8)	143.5	(150.3, 161.5)
δ 6.93 (1H, s)	103.1	(111.5,150.3, 143.9)
δ 6.85 (1H, s)	107.5	(143.5,149.8)
δ 6.27 (1H, d, *J* = 10)	113.5	(111.5, 161,5)
δ 6.15 (1H, s, -OH)	149.8	-
δ 3.96 (3H, s, -OCH_3)	56.6	(143.9)

Table 2. Antimicrobial activity of isolated compound against Xoo isolates

Source of Xoo isolate	Diameter of inhibition zone (mm)
TN 1	19 ± 0.33
TNRH 180	17 ± 0.58
CO 47	16.5 ± 0.00
CO 50	13 ± 0.58
ADT 39	18.5 ± 0.00
ADT 43	14.5 ± 0.29

Values are mean ± SE of three replicates

Table 3. Antioxidant activity of isolated compound along with standards

Samples	FRAP (mM Fe(II)/mg)	Metal chelating (µg EDTA/mg)	$ABTS^{+\cdot}$ (mM Trolox /mg)	Percentage inhibition		
				$DPPH^{\cdot}$	$OH^{\cdot}$	$O_2^{-\cdot}$
Compound	95.36 ± 1.40	1.16 ± 0.01	51.8 ± 0.7	31.8 ± 0.96	28.19 ± 0.63	42.08 ± 1.07
Rutin	291.09 ± 1.36	0.55 ± 0.01	86.6 ± 1.4	86.6 ± 1.03	60.04 ± 0.36	61.18 ± 1.95
BHT	325.43 ± 1.21	1.54 ± 0.01	101.5 ± 2.0	74.2 ± 0.79	45.77 ± 0.52	51.98 ± 1.91

Table 4. Protein docking studies with Xoo specific protein

Target protein	Compound	Binding energy (Kcal /mol)	Number of H-bonds	Interaction site Compound.....Protein
LipA (PDB-3H2G)	Tetracycline	-7.17	3	O.....H- Tyr 175
	6-hydroxy-7-methoxycoumarin	-6.48	2	O.....H-N Thr 88 O.....H- Ser 176 O.....H- Tyr 175 O.....H- Ser 176
FabV (PDB-3S8M)	Tetracycline	-9.28	4	H.....O- Phe 74
	6-hydroxy-7-methoxycoumarin	-7.14	2	O.....H-Ser 142 O.....H-N Phe 74 H.....O- Glu 75 H.....O-Val 274 O.....O-N-Val 274

that were assignable to one carbonyl, 4 quaternary carbons, 4 methine and 1 methoxy carbons. Presence of characteristic carbonyl at ´ 161.5 and the AB doublets with *J* value 10 Hz confirmed the compound is coumarin derivative (Table 1).

The ^{13}C-NMR shifts of compound were determined by heteronuclear single quantum coherence (HSQC) experiment. The HSQC of compound revealed the protons at d 6.27 and 7.60 were coupled with the carbons at d 113.5 (C-3),

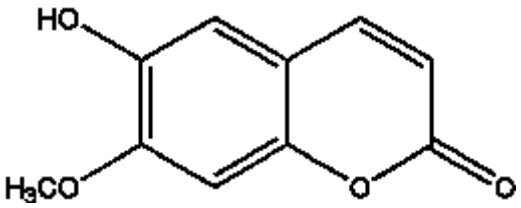


Fig.1. Chemical structure of isolated compound

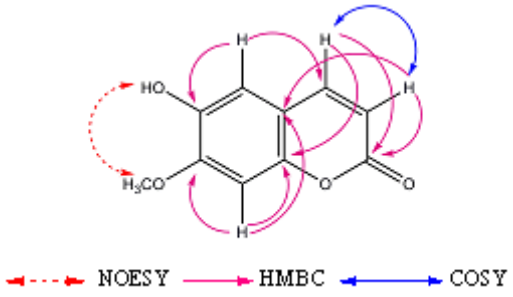


Fig. 2. Important 2D correlations of compound

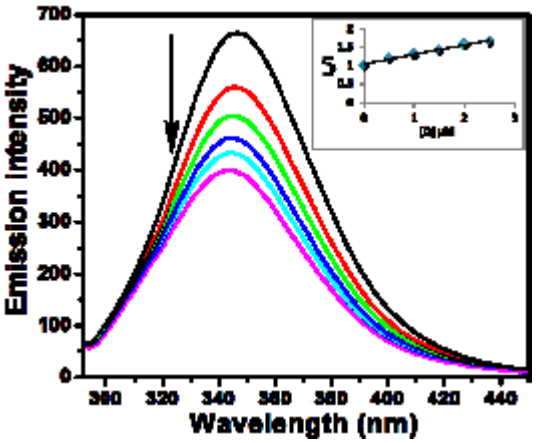


Fig. 3. The emission spectra of BSA in the presence of increasing amounts of compound (0, 0.5, 1, 1.5, and 2 µM). The inset shows Stern-Volmer plots of the fluorescence titration.

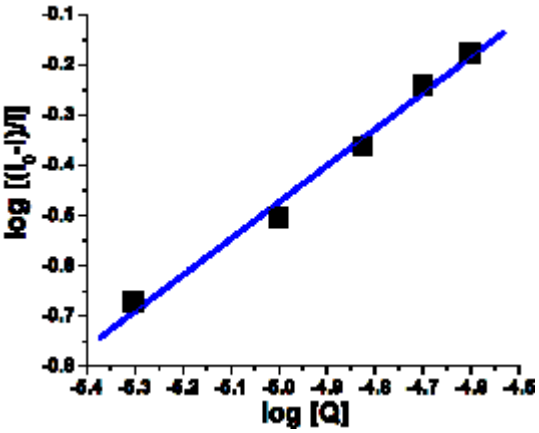


Fig. 4. Scatchard plots of the fluorescence titration of isolated compound with BSA

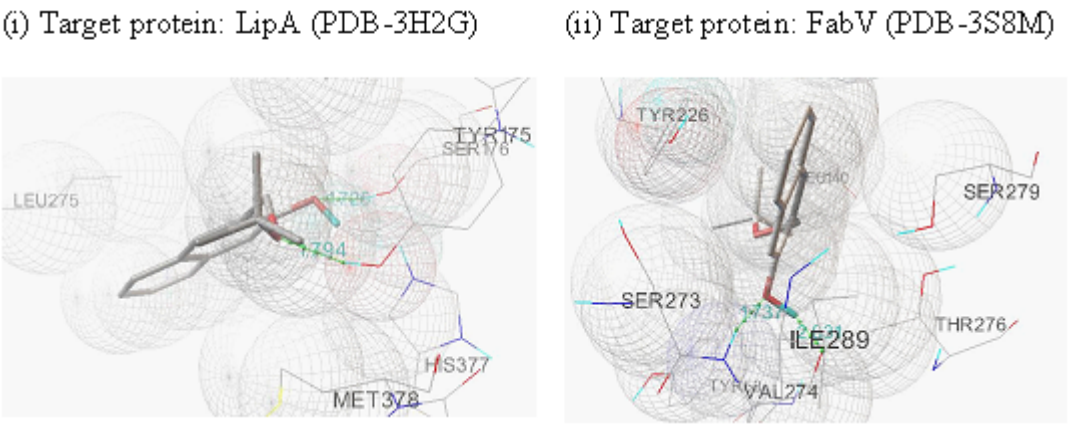
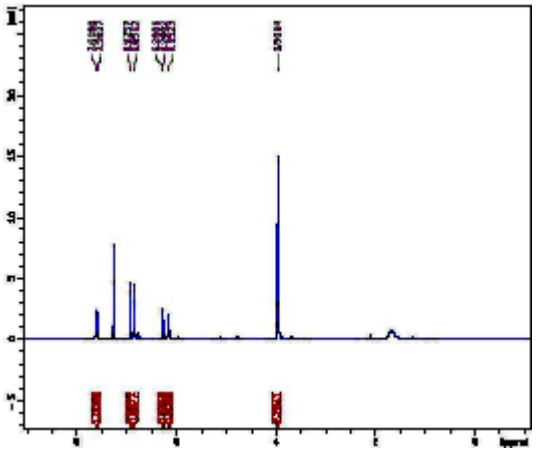


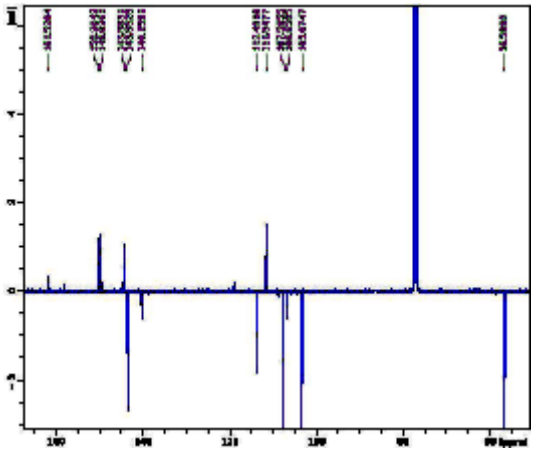
Fig. 5. Docking of isolated compound with receptor proteins (i) 3H2G and (ii) 3S8M in AutoDock software.

Supporting information (For review purpose)

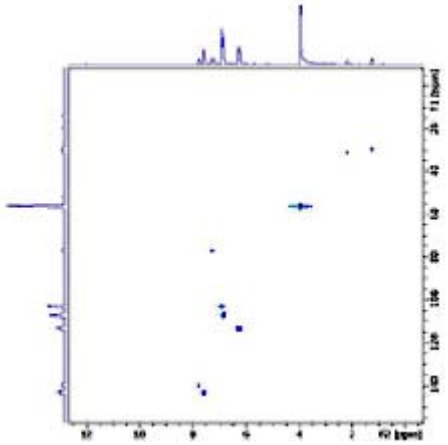
S1. ^{1}H NMR spectrum of compound



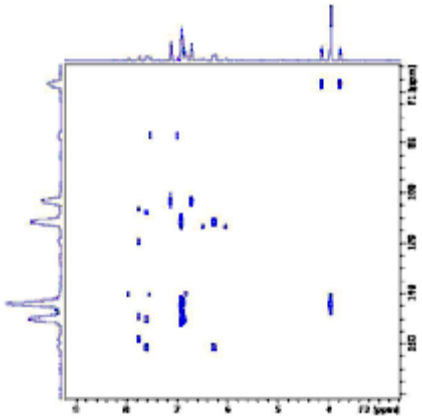
S2. ^{13}C NMR spectrum of compound



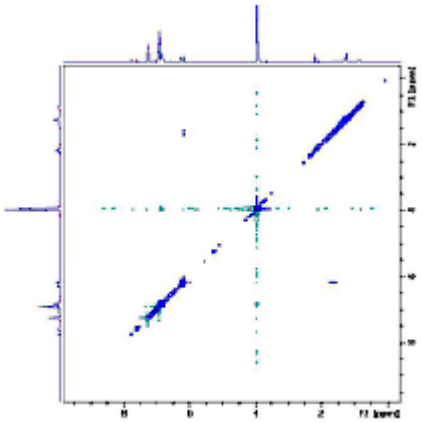
S3. HSQC spectrum of compound



S4. HMBC spectrum of compound



S5. NOESY spectrum of compound



and 143.5 (C-4) respectively (Table 1), while the proton resonating at d 6.93 and 6.85 coupled with the carbons at d 103.1 (C-8) and 107.5 (C-5) respectively. The position and the substitution patterns of hydroxyl and methoxy were determined by heteronuclear multiple bond correlation (HMBC) (Table 1), H-3 (´ 6.27) and H-4 (´ 7.60) showed cross peaks with d 111.5 (C-4a), d 161.5 (C-2) and d 150.3 (C-1a), d 161.5 (C-2) respectively. The HMBC spectrum also exhibited long range couplings of d 6.85 (H-5) and d 6.93 (H-8) with d 143.5 (C-4a), 149.8 (C-6) and d 111.5 (C-4a), 143.9 (C-7), 150.3 (C-1a) confirms the position of OH and OCH_3 at C-6 and C-7 respectively. This was further confirmed by the Nuclear Overhauser Effect Spectroscopy (NOESY) that exhibited association of C-6/OH with C-7/OCH_3 (Figure 2). Therefore the locus of hydroxyl and methoxy groups were confirmed at C-6 and C-7 and the proposed structure of compound were assigned as 6-hydroxy-7-methoxycoumarin.

Antimicrobial activity

The antimicrobial activities in terms of zone of inhibition against various isolates of Xoo were carried out for the isolated compound. The coumarin derivative produced a zone of inhibition of 13-19 mm against the tested Xoo isolates (Table 2). The highest activity of 19 mm inhibition zone was found against the Xoo isolate obtained from TN1 variety. Whereas the lowest activity of 13 mm zone of inhibition was observed for Xoo isolate obtained from CO50 variety. The tetracycline antibiotic produced an inhibition zone of 27-31 mm against the tested Xoo isolates. The variation observed in the antimicrobial activity against the Xoo pathogen is due to the existence of different resistance genes among the races of Xoo isolates.

Antioxidant activity

The antioxidant (FRAP and metal chelating) and radical scavenging ($ABTS^{+\cdot}$, $DPPH^{\cdot}$, $OH^{\cdot}$ and $O_2^{-\cdot}$) properties of isolated compound along with standards (rutin and BHT) were screened mainly by *in vitro* methods and their corresponding values are tabulated. In most of the antioxidant assays the isolated compound exhibited moderate antioxidant activity compared to standards rutin and BHT (Table 3). The compound exhibited good reducing power with the value of 95.36 (mM/mg) Equi. Ferrous ion, which is lower than the value observed for standards. The good chelating effect would be beneficial and removal of free state iron from circulation could be a promising approach to prevent oxidative stress-induced diseases. The metal chelating activity of the isolated compound is 1.16 (µg/mg) Equi. EDTA, which is more than that of value obtained for standard rutin. The total antioxidant activity of the isolated compound is analyzed by ABTS radical scavenging activity. The isolated compound exhibited an $ABTS^{+\cdot}$ assay value of 51.8 (mM/mg) Equi. Trolox, which was lower to the value observed for standards. The model of scavenging the stable $DPPH^{\cdot}$ radical is a widely used method to evaluate the free radical scavenging ability of various samples. The effect of antioxidants on $DPPH^{\cdot}$ radical scavenging was thought to be due to their hydrogen donating ability. The isolated compound exhibited a $DPPH^{\cdot}$ radical scavenging activity of 31.8%. Similarly the radical scavenging ability of the isolated compound is carried out for $OH^{\cdot}$ radicals. The percentage inhibition of $OH^{\cdot}$ radicals by the isolated compound is observed as 28.19% and compared to standard rutin which showed 60.04%. The superoxide radical scavenging activity exhibited by compound is 42.08%, which is lower than the value observed for standards rutin and BHT.

Protein binding studies

The effects of the compound on the fluorescence emission spectrum of BSA are shown in Figure 3. The addition of the isolated compound to the solution of BSA resulted in a significant decrease of the fluorescence intensity at 346 nm, up to 34% from the initial fluorescence intensity of BSA accompanied with blue shift (Figure 3). The arrow in the figure 3 shows the fluorescence quenching upon the increasing in concentration of the compound. In order to compare quantitatively, the binding strength of the compound, quenching constant (K_q), binding constant (K_b), and number of binding sites with BSA protein were determined using Stern-Volmer (Figure 3 inset) and Scatchard equation (Figure 4). The calculated value of n is 1 for the compound, indicating the existence of just a single binding site in BSA. The values of K_q ($2.26\pm0.15\times10^5\ M^{-1}$) and K_{bin} ($0.14\pm0.04\times105\ M^{-1}$) of the compound suggested that it has strong interactions with BSA.

Docking studies against Xoo specific protein

Docking of compound with specific proteins of interest, which is responsible for bacterial blight disease in rice were carried out. The possible conformation of compound along with their binding energy is shown in Table 4. The interaction site between compound and target protein is denoted as green color dots (Figure 5). The lower the binding energy indicates better docking of compound with the desired proteins. The docking capacity of compound is compared with a standard antibiotic Tetracycline. The binding energy of the compound with LipA protein was -6.48 Kcal/mol, which is very closer to the binding energy obtained for Tetracycline. Further the compound produced two hydrogen bonds with tyrosine and serine region of the target protein LipA. Whereas the compound produced a binding energy of -7.14 Kcal/mol with FabV protein, which is lower to the binding energy obtained for Tetracycline. The two hydrogen bonds produced with the valine residue of the target protein FabV. The antibiotic tetracycline exhibit good binding potential, which produced 3 and 4 hydrogen bonds with the target protein LipA and FabV respectively. Binding energies of the protein-ligand (compound) interactions are important to describe how the compound binds to the target macromolecule. Based on the binding energy and number of hydrogen bonds formed, it is inferred that isolated compound exhibit antimicrobial activity against receptor protein 3H2G and 3S8M.

DISCUSSION

Several members of the actinomycetes produce important secondary metabolites, including antibiotics, siderophore, enzyme and plant growth-promoting substances which may contribute to their host plants by promoting growth and enhancing their ability of withstanding the environmental stresses[25,26]. Over 55% of antibiotics have been isolated from genus *Streptomyces* and therefore this genus is one of several biological control agents which are widely studied and used to control various plant pathogens[27]. For instance, Prabavathy *et al.*,[28] reported that the effectiveness of *Streptomyces* sp. PM5 inhibit the mycelia growth of rice blast fungus *Pryricularia oryzae* and the rice sheath blight fungus *Rhizoctonia solani* was related to the production of aliphatic antifungal compounds (SPM5C-1 and SPM5C-2) which have lactone and ketone carbonyl units.

In the present investigation an antimicrobial metabolite against bacterial leaf blight pathogen of rice *Xanthomonas oryzae* pv. *oryzae* is isolated from the culture filtrate of *Streptomyces* sp. TC1. The isolated compound subjected to extensive spectroscopic studies and the structure of the compound is derived as 6-hydroxy-7-methoxycoumarin.

Coumarin and coumarin-related compounds have proved for many years to have significant therapeutic potential. They come from a wide variety of natural sources and new coumarin derivatives are being discovered or synthesised on a regular basis[29]. Although most of the natural coumarins in existence have been isolated from the higher plants, some members have been discovered in microorganisms. Some important coumarin members have been isolated from microbial sources e.g. novobiocin and coumermycin from *Streptomyces*, and aflatoxins from *Aspergillus* species[30].

de Souza *et al.*,[31] reported the antimicrobial activity of coumarin and their 45 other derivatives against strains of *Bacillus cereus* MIP 96016, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 by MIC and minimum bactericidal concentration (MBC) assay. Bacterial susceptibility to coumarins was evaluated and those that were active exhibited MIC values ranging from 62.5 to 2000 ¼g/ml. Among the active compounds, osthenol showed the most potent activity with MIC of 62.5 ¼g/ml.

In the present study the isolated 6-hydroxy-7-methoxycoumarin evaluated against various isolates of bacterial leaf blight pathogen Xoo by agar well diffusion assay and found to exhibit an inhibition zone of 13-19 mm. The improved rice varieties released in India, though had a certain degree of resistance to bacterial blight, became susceptible due to emergence of new races of the pathogen[32]. This may be due to existence of variability in the pathogen and emergence of new pathotypes[33]. Each pathotypes posses varied levels of susceptibility to antimicrobial compounds due to their resistance genes[34].

The production of reactive oxygen species (ROS) in bacteria attack, almost all cell components, including DNA, protein and lipid membrane. This ROS, which includes the superoxide anion, hydrogen peroxide and the hydroxyl radical in bacteria, is mainly related to respiratory chain activity[35]. Recent studies have indicated that a radical-based approach is needed for the treatment of free-radical-induced diseases[36]. Free radicals are chemical species containing one or more unpaired electrons that makes them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. In recent years much attention has been devoted to natural antioxidant and their association with health benefits. There are several methods available to assess antioxidant activity of compounds[37].

In the present study the antioxidant activity of the coumarin compound is analyzed by six different *in vitro* assays. The antioxidant activity is also compared with the standard antioxidants rutin and BHT. The compound exhibited moderate antioxidant activity in the tested assays. Kostova *et al.*,[38] reported a detailed review on the antioxidant properties of courmain and their derivatives obtained from natural and synthetic sources.

The interaction of compound with protein moieties were studied by protein quenching assay and docking studies. The results obtained from the BSA protein binding experiments of isolated compound revealed that the binding of compound with BSA is mainly due to the hydrophobic and electrostatic interactions. So, the strong interaction between the compound and BSA suggested that this can easily be stored in protein and will be released in desired targets.

Docking small, mostly organic compounds to proteins is relevant to both understanding biological processes and designing drugs. The aim of molecular docking is to achieve an optimized conformation for both the protein and ligand and relative orientation between protein and ligand such that the free energy of the overall system is minimized[39].

The docking study results clearly indicate that compound were having the capacity to bind the FabV protein responsible for fatty acid elongation in Xoo and can inhibit the growth of the organism by inhibiting the cell wall synthesis. The another protein LipA is a secretory virulence factor of Xoo, implicated in degradation of rice cell walls and the concomitant elicitation of innate immune responses, such as callose deposition and programmed cell death. The degradation of rice cell wall favours the entry of pathogen and emergence of systemic infection. The binding of the compound with LipA protein prevent the entry of Xoo pathogen in rice plants thereby prevent the damage caused by bacterial leaf blight. The binding energy obtained for the compound with the rice cell wall degrading protein LipA is very closer to the value exhibited by standard antibiotic tetracycline. Similarly there are proteins like FabH[40], XoMCAT[41] which responsible for bacterial blight disease in rice are considered to be a good antimicrobial target for bacterial blight disease management and control.

CONCLUSION

In summary, we have identified biologically active 6-hydroxy-7-methoxycoumarin from the culture filtrate of *Streptomyces* sp. TC1. The antimicrobial property of the isolated compound against bacterial leaf blight pathogen is well supported by the antimicrobial assay and docking studies. Based on docking studies, the compound also has the capacity to bind with the rice cell wall degrading protein produced by Xoo strain and preventing them to cause systemic infection. These results strongly recommend that the *Streptomyces* sp. TC1 can be exploited as a biocontrol organism for bacterial leaf blight disease management in rice.

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REFERENCES

1. Tanaka, Y., Omura, S. Agroactive compounds of microbial origin. *Annu. Rev. Microbiol.,* 1993; **47**: 57-87.

2. Xu, L., Li, O., Jaing, G.L. Diversity of soil actinomycetes in Yunnan China. *Appl. Environ. Microbiol.*, 1996; **62**: 244-248.
3. Miyadoh, S. Research on antibiotic screening in Japan over the last decade. A producing microorganism approach. *Actinomycetologica*, 1993; **9**: 100-106.
4. Hosoya, Y., Adachi, H., Nakamura, H., Nishimura, Y., Naganawa, H., Okami, Y. The structure of diphenazithionin, a novel antioxidant from *Streptomyces griseus* ISP 5236. *Tetrahedron Lett.*, 1996; **37**: 9227-9228.
5. Chang, H.B., Kim, J. Antioxidant properties of dihydroherbimycin A from a newly isolated *Streptomyces* sp. *Biotechnol. Lett.*, 2007; **29**: 599-603.
6. Kim, K.J., Kim, M.A., Jung, J.H. Antitumor and antioxidant activity of protocatechualdehyde produced from *Streptomyces lincolnensis* M-20. *Arch. Pharmacal. Res.*, 2008; **31**: 1572-1577.
7. Shimizu, M., Nakagawa, Y., Sato, Y. Studies on endophytic actinomycetes (I) *Streptomyces* sp. Isolated from rhododendron and its antifungal activity. *J. Gen. Plant Pathol.*, 2000; **66**(4): 360-366.
8. El-Abyad, M.S., El-Sayed, M.A., El-Shanshoury, A.R., El- Sabbagh, S.M. Towards the biological control of fungal and bacterial diseases of tomato using antagonistic *Streptomyces* spp. *Plant and Soil*, 1993; 149: 185-195.
9. Gnanamanickam, S.S., Priyadarisini, V.B., Narayanan, N.N., Preeti, V., Kavitha, S. An overview of bacterial blight disease of rice and strategies for its management. *Curr. Sci.*, 1999; **77**(11): 1435-1443.
10. Mew, T.W. Current status and future prospects of research on bacterial blight of rice. *Annu. Rev. Phytopathol.*, 1987; **25**: 359-382.
11. Weller, D.M. Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. *Ann. Rev. Phytopathol.*, 1988; **26**: 379-407.
12. Vasudevan, P., Kavitha, S., Priyadarisini, V.B., Babujee, L., Gnanamanickam, S.S.: Biological control of rice diseases. In: *Biological control of crop diseases* (Gnanamanickam SS, ed). New York: 2002; pp 11-32.
13. Perez, C., Paul, M., Bazerque, P. An antibiotic assay by the agar well diffusion method. *Acta Bio. Med. Exp.*, 1990; **15**: 113-115.
14. Pulido, R., Bravo, L., Sauro-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/ antioxidant power assay. *J. Agric. Food Chem.*, 2000; **48**: 3396-3402.
15. Dinis, T.C.P., Madeira, V.M.C., Almeida, L.M. Action of phenolic derivatives (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.*, 1994; **315**: 161-169.
16. Re, R., Pellegrini, N., Proteggente, A., Panala, A., Yang, M., Rice-Evans, C. Antioxidant Activity Applying an Improved ABTS Radical Cation Decolorization Assay. *Free Radic. Biol. Med.*, 1999; **26**: 1231-1237.
17. Campodonico, P., Barbieri, E., Pizarro, M., Sotomayor, C.P., Lissi, E.A. A comparison between total phenol content of wines and their TRAP values measured by the bleaching of ABTS radical cations. *Bol. Soc. Chil. Quim.*, 1998; **43**: 281-285.
18. Blios, M.S. Antioxidant determinations by the use of a stable free radical. *Nature*, 1958; **26**: 1199-1200.
19. Klein, S.M., Cohen, G., Cederbaum, A.I. Production of formaldehyde during metabolism of dimethyl sulphoxide by hydroxyl radical generating system. *Biochem.*, 1991; **20**: 6006-6012.
20. Nishikimi, M., Appaji, N., Yagi, K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochem. Biophys. Res. Commun.*, 1972; **46**: 849-854.
21. Raja, D.S., Bhuvanesh, N.S.P., Natarajan, K. Biological evaluation of a novel water soluble Sulpher bridged copper (II) thiosemicarbazones complex. *Eur. J. Med. Chem.*, 2011; **46**: 4584-4594.
22. Schames, J.R., Henchman, R.H., Siegel, J.S., Sotriffer, C.A., Ni, H., McCammon, J.A. Discovery of a novel binding trench in HIV integrase. *J. Med. Chem.*, 2004; **47**(8): 1879-81.
23. Aparna, G., Chatterjee, A., Sonti, R.V., Sankaranarayanan, R. A cell wall-degrading esterase of *Xanthomonas oryzae* requires a unique substrate recognition module for pathogenesis on rice. *Plant Cell*, 2009; **21**: 1860-1873.
24. Li, H., Zhang, X., Bi, L., He, J., Jiang, T. Determination of the crystal structure and active residues of FabV, the enoyl-ACP reductase from *Xanthomonas oryzae*. *Plos One*, 2011; **6**: 26743-26743.
25. Khamna, S., Yokota, A., Lumyong, S. Actinomycetes isolated from medicinal plant rhizosphere soils: Diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. *World J. Microbiol. Biotechnol.*, 2009; **25**: 649-655.

26. Qin, S., Xing, K., Jiang, J.H., Xu, L.H., Li, W.J. Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Appl. Microbiol. Biotechnol.,* 2011; **89**: 457-473.
27. Embley, T.M., Stackebrandt, E. The molecular phylogeny and systematics of the actinomycetes. *Ann. Rev. Microbiol.,* 1994; **48**: 257-289.
28. Prabavathy, V.R., Mathivanan, N., Murugesan, K. Control of blast and sheath blight diseases of rice using antifungal metabolites produced by *Streptomyces* sp. PM5. *Biol. Control,* 2006; **39**: 313-319.
29. Lacy, A., Kennedy, R.O. Studies on coumarins and coumarin-related compounds to determine their therapeutic role in the treatment of cancer. *Curr. Pharma. Design,* 2004; **10**: 3797-3811.
30. Cooke, D., Fitzpatrick, B., Kennedy, R.O., McCormack, T., Egan, D.: Coumarins - multifaceted molecules with many analytical and other applications. In: *Coumarins: biology, applications and mode of action* (Kennedy RO, Thornes RD, eds.). United Kingdom: Wiley & Sons, 1997; pp 303-332.
31. de Souzaa, S.M., Monacheb, F.D., Smania Jr, A. Antibacterial activity of coumarins. *Z. Naturforsch.,* 2005; 60c: 693-700.
32. Khush, G.S., Mackill, D.J., Sidhu, G.S.: Breeding rice for resistance to bacterial blight. In: *Proc. Int. Workshop on bacterial blight of rice*. IRRI, The Philippines: 1989; pp 207-217.
33. Goel, R.K., Kaur, L., Saini, G.S. Effectiveness of different *Xa* genes against *Xanthomonas oryzae* pv. *oryzae* population causing bacterial blight of rice in Punjab (India). *Rice Genet. Newsl.,* 1998; 15: 131.
34. Leach, J.E., Leung, H., Nelson, R.J., Mew, T.W. Population biology of *Xanthomonas oryzae* pv. *oryzae* and approaches to its control. *Curr. Opin. Biotechnol.,* 1995; **6**: 298-304.
35. Lushchak, V.I. Oxidative stress and mechanisms of protection against it in bacteria. *Biochem.,* (Mosc.) 2001; **66**: 476-89.
36. Cleveland, J.L., Kastan, M.B. Cancer: a radical approach to treatment. *Nature,* 2000; **407**: 309.
37. Ali, S.S., Kasoju, N., Luthra, A., Singh, A., Sharanabasava, H., Sahu, A. Indian medicinal herbs as sources of antioxidants. *Food Res. Intl.,* 2008; **41**: 1-15.
38. Kostova, I., Bhatia, S., Grigorov, P., Balkansk, S., Parmar, V.S., Prasad, A.K. Coumarins as antioxidants. *Curr. Med. Chem.,* 2011; **18**(25): 3929-51.
39. Kitchen, D.B., Decornez, H., Furr, J.R., Bajorath, J. Docking and scoring in virtual screening for drug discovery: methods and applications. *Nature reviews Drug discovery* 2004; **3**(11): 935-49.
40. Huynh, K.H., Natarajan, S., Song, N.H., Ho Ngo, P.T., Ahn, Y.J., Kim, J.G. Cloning, expression, crystallization and preliminary X-ray crystallographic analysis of ²-ketoacyl-ACP synthase III (FabH) from *Xanthomonas oryzae* pv. *oryzae*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.,* 2009; **65**(Pt 5): 460–462.
41. Natarajan, S., Kim, J.K., Jung, T.K., Doan, T.T., Ngo, H.P., Hong, M.K. Crystal structure of malonyl CoA-Acyl carrier protein transacylase from *Xanthomonas oryzae* pv. *oryzae* and its proposed binding with ACP. *Mol. cells,* 2012; **33**(1):19-25.