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RESEARCH ARTICLE

Devulging the treasure of hydroxamate siderophore on keratinocyte migration; its implication for wound healing

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Abstract

Fungi, fantastic organisms that feed us, heal us, divulge secrets of the universe, and could help save the human life today. An enormous of fungi are being to produce and excrete desferi-siderophore complexes under the iron-limiting conditions. It is considered to be a potentially powerful method for a rational design of the therapeutic agents against the drug resistant micro-organisms and such active compounds to promote the angiogenic activity of cells. A ferrated siderophore production from a culture of *Penicillium chrysogenum* isolated from soil was screened through chrome azurol sulphonate (CAS) assay. It was isolated, purified and characterized. The chemical characterization revealed that it was hydroxamate type and it was confirmed through UV spectrophotometer, FTIR and ESI-MS. The influence of growth medium, incubation time and temperature for growth of the *P.chrysogenum* with considerable siderophore producing potential was investigated. The optimal temperature for mycelial growth was obtained at 25 °C and modified Grimm Allen iron fortified medium exhibited more siderophore production. We further investigated the molecular characterization of siderophore coding gene L-ornithine encoded by *sid 1* gene expression with iron limited and iron supplemented grown cultures. This Penicillial trihydroxamate highly expressed in human keratinocyte cell line (HaCat) is known for its striking ability to promote cell adhesion and cell proliferation even under glucotoxic (30 mM) condition. It also increases the synthesis of reactive oxygen species (ROS) which stimulates the activity of growth hormones which in turn play a major role in the process of wound healing. This study was designed to anticipate the role of trihydroxamate in keratinocyte migration and proliferation along with its mRNA expression. The results divulged that trihydroxamate promotes keratinocyte migration and to induce proliferation. The mRNA expression study revealed that the trihydroxamate enhance the growth hormones expression in cells. This trihydroxamate siderophore has a significant role in wound healing specifically during impaired angiogenic condition by promoting keratinocyte migration and to induce growth factors activity.

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Introduction:-

A chronic wound is any interruption in the continuity of the skin and integrity of the tissue that requires a prolonged time to heal, does not heal, or recurs [1]. The process of wound healing comprises events of cell migration, cell proliferation, and extracellular matrix (ECM) deposition. Normal wound healing requires proper circulation, nutrition, immune status, and avoidance of negative mechanical forces. The process of tissue repair usually takes 3–

14 days to complete and has three distinct phases: inflammation, proliferation, and remodeling with wound contraction [2]. Wounds gain 80 % of their final strength in the first 3 weeks of normal wound healing through collagen deposition, remodeling, and wound contraction [3]. When any of the components of the wound healing process is compromised, healing may be delayed.

Furthermore, the absence of keratinocyte migration at the wound edge is a critical defect related to the clinical phenotype of chronic nonhealing wounds, such as diabetic ulcers [4]. There are multiple factors in the diabetic wound including increased apoptosis, decreased vascular recovery, an aberrant inflammatory response and delayed cellular turnover contribute to the impaired wound healing. Moreover, the production of reactive oxygen species (ROS) from the inflammatory cells and other cells in the wound is required for defense against invading bacteria. The latest therapeutic modalities for managing diabetes foot are now available to Indian patients. The vacuum assisted closure (VAC) therapy treatment modalities cost Rs 50,000–57,000 for 3 weeks. Fixator treatment costs more than Rs 1 lakh, growth factors costs upto Rs 1200/tube of 7.5 g. Therefore these modalities cannot reduce the number of amputations and can be used only sparingly in India. Thus, the economic costs associated with chronic wounds are enormous and involves hospital expenditure, disability, low productivity, and a loss of independence. Hence, people need alternative bioactive compounds for effective treatment of the diabetic wound in a cost effective manner. A drug used to remove iron from the body could help doctors fight one of diabetes' cruelest complications [5].

Penicillium chrysogenum is an important filamentous fungus in the medical industry for producing penicillin and derived β lactam antibiotics. It also produces a low molecular weight iron chelating compounds termed siderophores to scavenge iron from the environment reeling under conditions of an iron stress. Fungi have also been studied for their siderophores and their role in the iron transport process [6]. A variety of fungi are known to produce and excrete desferi-siderophore complexes under the iron-limiting conditions. Siderophore-specific transport systems utilize such complexes and stand for the energy consuming systems as it could be inferred from their sensitivity to respiratory inhibitors, uncouplers and the changes they bring about on the membrane potential and are recognized as the structure of the stereo chemical configuration of the siderophore molecule. A new hydroxamate type siderophore was synthesized and characterized in terms of its acid-base behaviour; they form a stable ferric complex and are detected in *Candida albicans* by the spectrophotometric methods [7]. Molecular mechanical methods revealed most probable structure of the ferric complex. The Biscatechol hydroxamates chelators of a similar structure catalyzed a set of acylation reactions and were tested for their siderophoric activity in the various receptor-deficient mutants of *Escherichia coli.* The most of the fungi display the occurrence of specific mechanisms for iron acquisition from the hosts they infect for their own survival. In 1952, Neilands [8] reported ferrichrome, the first chemically defined cyclic hexapeptide hydroxamate type siderophore then the same type of siderophores were reported from many basidiomycetes and ascomycetes including *Penicillium* and *Aspergillus* sp. Since, little is known on the siderophore production in *P.chrysogenum* in general and in particular this study has been undertaken to investigate (1) the production and optimization of conditions for mass production of siderophores, (2) its chemical characterization and quantification, (3) molecular confirmation on siderophore coding genes in *Penicillium chrysogenum* and (4) Assessment of invitro wound healing ability of siderophore through keratinocyte migration, proliferation, reactive oxygen species production and mRNA expression.

Materials and methods:-

2.1. Fungal strain and growth conditions:-

The fungal isolate was identified as *Penicillium chrysogenum* through microscopical (Fig. 1) and morphological confirmation. It is a common inhabitant of soil. The culture was kept on potato dextrose agar slants. The siderophore production was tested by chrome azurol sulphonate [8] agar plate assay, is a universal method to detect siderophore production. To determine the type of siderophore production culture supernatant was used. The presence of hydroxamate siderophore was checked according to Atkin et al. [9] method measured the absorbance at 480 nm. Desferol was used as standard. The presence of catechol type siderophore was checked according to Arnow's assay [10]. The type of siderophore was also be detected through thin layer chromatography with butanol: acetic acid: water (6:1:1) solvent system.

2.2. Optimization of conditions for siderophore production:-

Four media components were tested for growth of the organism for siderophore production. Fries basal medium [11], King B medium (60786-Fluka King Agar B), Vogel N medium [12] and modified Grimm Allen medium [13] were studied for the growth and siderophore production. Likewise, the effect of temperature (4, 15, 20, 25, 30, 35

and 40 °C) and the effect of incubation time from 0 to 24 days were also been monitored. The culture supernatant was collected to measure the growth at OD 600 nm and the iron perchlorate assay was used to determine the approximate amount of siderophore at OD 450 nm.

2.3. Effect of iron concentration on *sid1* gene expression:-

Iron concentration of a medium is critical to whether or not siderophore biosynthesis genes are expressed. After the optimized growth was reached the total RNA was extracted according to trizol method from the cells grown at 10 μM $FeSO_4$ supplemented and 1 μM $FeSO_4$ limited medium. The cDNA was constructed and 1500 bp fragment of a gene encoded for L-ornithine was amplified with primer 5′ ACTACAGTGTTGTCCGCCTG 3′ and 3′ ATCGATTGGACCATCTCGCC 5′. The amplified PCR product was electrophoressed by 2 % agarose gel electrophoresis to confirm the siderophore production.

2.4. Purification of siderophore:-

The culture was grown in large scale on GA medium for 14 days at 25 °C on a rotary shaker at 70 rpm. After incubation the culture supernatant was collected by centrifuging at 7000×*g* for 30 mins. The supernatant was then acidified to pH 2.0 with 6 M Hcl. The acidified supernatant was passed through the XAD-2 amberlite column which binds cyclic compounds and the flow was collected. All fractions were tested for their siderophore content using the iron perchlorate assay. The positive fractions were collected and air dried in Buchi R200 rotary evaporator with the temperature set to 70 °C. The concentrated sample was then loaded into the sephadex LH20 column which to separate compounds based on molecular weight and eluted with methanol. The positive fractions were combined and evaporated to dryness using rotary evaporator. The dried sample was then redissolved with millipore water for further analysis.

2.5. High performance liquid chromatography:-

HPLC analysis was performed with concentrated siderophore using a Biorad Biologic Duoflow HPLC system with water 7.8 × 300 mm Novopak HR C18 hydrophobic column as the stationary phase and deaerated, filtered ddH_2O and filtered 90 % acetonitrile as mobile phase. The UV detector was set at 220 nm to monitor the ferric hydroxamate complexes. The partially purified sample was run through HPLC; the fraction of siderophore was eluted and concentrated.

2.6. Chemical characterization:-

The HPLC eluted sample was used for various chemical analysis. A spectral scan was taken (300–700 nm) to determine hydroxamate type siderophore was dihydroxamate or trihydroxamate [14].

2.7. Aminoacid analysis:-

The purified sample was hydrolysed with 6 M Hcl or 6 M NaOH. Then it was run with standard aminoacids of alanine, serine, tryptophan, glycine and tyrosine on silica gel plates using different solvent systems. These included methanol: 0.1 M ammonium acetate (60:40), acetonitrile: 0.1 M ammonium acetate (60:40), and n-propanol: ddH_2O (70:30) [15].

2.8. FTIR analysis:-

Purified 5 mg of slurry with KBr at 25 °C was pressed into a disk. The FTIR spectrum was recorded using FT/IR-4100typeA model.

2.9. NMR spectrum:-

All NMR spectra were collected at 298 K on a Bruker AVANCE 500 MHz instrument equipped with a triple resonance probe and triple axis gradients. All spectra were recorded using a 15N-labeled sample of the siderophore dissolved in D_2O and the solvent signal was used as internal reference for all 1H and 13C spectra; 15N spectra were referenced. All spectra were processed and analyzed using MestReNova software.

2.10. ESIMS analysis:-

Mass spectrometric analyse was carried out using Q-Star quadrupole TOF mass spectrometer in positive ion mode (Applied Biosystems). ESI-MS analyse was carried out using a NanoES spray capillaries. The electrospray voltage was set at 1.8 kV. The purified sample was used to inject into the ESI-MS was prepared in 30 % acetonitrile and 1 % TFA.

2.11. Cell culture:-

Immortalized human epidermal keratinocyte (HaCaT) was acquired from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Dulbecco's modified eagle medium: Ham's medium (Gibco, India) (1:1) supplemented with 10 % fetal bovine serum (PAN) and 1× antibiotic antimycotic (Gibco, India). The cells were maintained at 37 °C in a humidified 5 % CO_2 incubator in 25 cm two culture flasks.

2.12. Cell viability assay:-

Cytotoxicity was analyzed using the MTT [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide] assay with 24 and 48 h conditions in CO_2 incubator. The viability of cells was more than 80 % (IC 80) of concentration was selected for the further analysis. The cell viability was calculated by the following formula

$$\text{Survival rate}(\%) = \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{absorbance of negative control} - \text{absorbance of blank}} \times 100$$

2.13. Scratch wound assay:-

Eight different conditions were used for this study (Table. 1) Equal density of 2×10^5 cells (HaCaT) per well was seeded in a six well tissue culture plate and allowed to become confluent 48 h in a CO_2 incubator. A scratch wound was created on the monolayer of cells using a 200 µl pipette tip. The cells were then washed twice with warm PBS and fresh medium without serum containing different concentrations of hydroxamate siderophore were added in triplicates. The migration of the cells was captured using a phase contrast microscope (Leica Microsystems, Germany) at a time interval of 2 h and the wound area was measured using image J46 software. The percentage of wound recovered and migration rate were calculated using the formula given below. After the study period cells were fixed and observed for lamellipodia formation in a phase contrast microscope (Leica Microsystems, Germany).

$$\text{Percentage of wound area recovered} = \frac{\text{Initial scratch area} - \text{migrated cell surface area}}{\text{Total cell surface area}} \times 100$$

$$\text{Migration rate}(\mu m/hr) = \frac{\text{Initial scratch area} - \text{migrated cell surface area}}{\text{Total hours taken for recovered}}$$

2.14. Phalloidin staining:-

The characteristic lamellipodia formation of the migrating cells was identified by staining the cytoskeletal actin filaments. After treating the HaCaT cells with hydroxamate (60 µg/ml) overnight, cells were washed twice with PBS and fixed with 3.7 % formaldehyde. The fixed cells were then washed, extracted with 0.1 % tritonX 100 (Sigma-Aldrich, USA) and blocked with 1 % BSA. The cells were later washed twice with PBS and stained with 200 µl of 1× Oregon Green H488 Phalloidin (Invitrogen, USA) for 1 h in a dark humidified chamber. For control cells same procedure was performed excluding hydroxamate treatment and the same experiment was followed for glucotoxicity with 30 mM glucose was added to the well. The excess stain was removed by washing with PBS and images were captured using a fluorescence microscope (Leica Microsystems, Germany).

2.15. Cell proliferation assay:-

The proliferative potential of trihydroxamate on HaCaT cells was assessed by MTT assay. Equal density of 12×10^3 cells/well was seeded in a 96 well tissue culture plate. Following overnight incubation, fresh medium without serum containing different concentrations of hydroxamate siderophore were added in triplicates. After 24 and 48 h the cells were treated with MTT (0.5 mg/mL in PBS) for 4 h at 37 °C. The formazan complex formed by the live cells was dissolved and measured colorimetrically at 570/630 nm using a micro plate reader (Bio-Rad, USA).

2.16. Assessment of reactive oxygen species production:-

The reactive oxygen species production was carried out in multimode plate reader (Perkin Elmer's, Enspire). The cells along with different concentrations of trihydroxamate siderophore were treated with glucotoxic conditions in 96 well tissue culture plates. Continuing overnight incubation the cells were washed thrice with HEPES buffer. The washed cells were then treated with 20 µl/ml of 10 µM dichloro dihydro fluorescein diacetate acetyl ester (DCFDA) florescent dye. The plate was wrapped with aluminium foil and kept for an 1 h in CO_2 incubator. After incubation the cells were washed thrice with HEPES (pH 7.4) buffer and scrapped. They were transferred to a full dark 96 well plate and measured OD at 485/535nm for 0–15 min at 1 min interval. The phormol 12 myristate 13 acetate (PMA) was added at 1 min of reading to stimulate the ROS.

2.17. Evaluation of mRNA expression:-

Total RNA was extracted from 90 % confluent HaCaT cells using TRIzol method. The extracted RNA was quantified using Nanodrop 2000 (Thermofischer, USA), and 2 mg of total RNA was converted to cDNA. 1 µg of total RNA was used for the reverse transcription. Target genes were detected by realtime PCR with Fast Start universal SYBR GREEN MASTER (Roche.). The sequences of the primers were as follows: Human FSTL1 forward, 5′ TGGACTGTAACCGCTG TGTC 3′ and reverse 5′ AGCCCCAGAGCACCATTT AC 3′; FGF2 forward, 5′ CCACCTA TAATTGGTCAAAGTGGT 3′ and reverse 5′ TCATCAGTTACCAGCTCCCC 3′. BLT2 forward, 5′ TGTGATAGGCACAGGACAGG 3′and reverse 5′ GTTCAGGGGTGGGG CAAAAC 3′; MMP9 forward, 5′ TCTATGGTCCTCGCCCTGAA 3′ and reverse 5′ TTGTATC CGGCAAACTGGCT 3′; FOX1 forward 5′ TTATTCTCTCCCGCCCTCCT 3′ and reverse 5′ AGCGCAAGGACTCTCAATCC 3′; INTα3 forward 5′ ATGGCAAGTGGCTGCTGTAT 3′ and downstream region 5′ CTCAGTAGTCGTCGGTCAGC 3′; INTβ6 forward 5′ ATCGGTC TGCACAGCAAGAA 3′ and reverse 5′ GGGTATCACACCTTTCGCCA 3′; EGF forward 5′ GCCCCAATCCAAGGGTTGTA 3′ and reverse 5′ GCCCTGGGGCATC TTTTACT 3′; TGFα forward 5′ GAGAGTGGAGAGAAGCGAGT 3′ and downstream region 5′ CGGACAGT CCCCTCTTT GTC 3′; CCL2 forward 5′ TTCCCCTAGCTTTCCCCAGA 3′ and downstream region 5′ TCCCAGGGGTAGAACTGTGG 3′. PDGF1 forward 5′ GGTCGCTCCTGAAGC CAG 3′ and reverse 5′ GGAGGAGAAACAGGGAGTGC 3′; VEGF forward 5′ TCACC AAGGCCAGCACATAG 3′ and reverse 5′ GAGGCTCCAGGGCATTAGAC 3′. Gene expression levels were determined with the ΔΔCT method after normalization to the expression level of the standard housekeeping gene β actin in all experiments.

Results:-

3.1. Detection of siderophore:-

The chrome azurol sulphonate (CAS) assay was used to detect siderophore production in *P.chrysogenum*. In the CAS agar plate, an orange halo was around a disc of mycelium (Fig. 2) and a blue colour CAS solution was turned to orange red colour solution when culture supernatant was added. They were indicated siderophore production in *P.chrysogenum.* The type of siderophore was assessed through iron perchlorate assay and Arnow's assay. The formation of dark brownish red colour was indicated hydroxamate type siderophore in iron perchlorate assay and the absorbance was measured at 480 nm (Table. 1). The Arnow's assay was repeatedly indicated negative result for catechol type siderophore.

3.2. Optimization of conditions for siderophore production:-

Potato dextrose agar medium was used in the preliminary characterization of siderophore production. However the media with different conditions were needed to be optimized to achieve maximum siderophore production. There was Fries basal medium [16], King B medium (60786-Fluka King Agar B), Vogel N medium [7] and modified Grimm Allen medium [3] were evaluated with various temperatures and incubation days were carried out for the growth and siderophore production. Cultures were grown in modified Grimm Allen medium was showed highest growth rate at 25°C for 14 days incubation (Additional file 1: Figs. S1, S2, S3). The growth rate was measured at OD 600 nm and the estimation of siderophore was measured at OD 480 nm.

3.3. *sid1* gene expression:-

The *sid1* gene encodes L-ornithine N^5 oxygenase. *sid1* catalyses the hydroxylation of L-ornithine as the first step of ferrichrome siderophore biosynthesis. We have examined the *sid1* gene expression in the cultures were grown in different concentrations of iron supplemented conditions. The 10 µM $FeSO_4$ supplemented condition was not expressed (data not shown). The gene was expressed in 0.5 and 5 µM conditions.

3.4. Purification of siderophore:-

Once the growth conditions had been optimized, it was possible to produce maximum amounts of siderophore by *P.chrysogenum* was grown in batch cultures. The culture supernatant was first purified through XAD2 column chromatography with methanol solvent. The yellow colour fraction was collected and tested for its siderophore content using iron perchlorate assay (Fig. 3). The purified fraction was further purified through sephadex LH20 column. The concentration of the siderophore was estimated 100–130 mg present in the sample. The HPLC chromatogram was generated from high to low compounds. The siderophore peak was arised in 2.453 in high range (Fig. 4a). The elute was collected from that range for several times. The collected sample was tested for their siderophore content on silica gel plates by using the solvent system was butanol: acetic acid: water in the ratio of 6:1:1. A single spot was visualized in TLC plate under broad spectrum of UV range (Fig. 4b).

3.5. Biochemical assessment of siderophore:-

The spectral scan analysis (300–700 nm) of purified sample revealed that the purified siderophore was a trihydroxamate type (400–500). Many of the siderophores contain one to more aminoacids in their structure. Three different solvent systems were used. Inwhich, acetonitrile: 0.5 M ammonium acetate (6:4) showed good chromatogram (Table 2). The other solvent systems were completely smeared on the silica gel plates. The plates showed spots on the tryptophan, L-ornithine and serine. The three aminoacids presence were also confirmed the siderophore production in *P.chrysogenum*. The L-ornithine is a vital aminoacid in siderophore biosynthesis pathway.

The FTIR spectrum showed a strong broad band at 3400 cm^{-1} indicated OH stretching, while a weaker, sharper band indicated N–H stretching at 1600 cm^{-1}. The strong absoption band at 1647 cm^{-1} in the IR spectrum was indicated a C=O group attached to an aromatic group (Table 3).

^{1}H and 13C NMR spectra of the purified siderophore revealed to elucidate the primary structure of the compound. The amide and amino protons were identified in this spectrum. The aminoacids sub chains were assigned with NH protons. The hydroxyl groups were identified at 5.11 and 4.33 ppm (Table 4). The spectrum contains peaks 7.22 and 1.26 ðppm for alanyl NH and CH_3 unique. In ^{1}H–NMR spectrum showed methyl group and singlet signal at 1.72 ppm due to the methyl group and a singlet at 4.33 ppm *N*-methyl group.

The ESIMS spectrum revealed to determine molecular weight based on the peaks obtained in the mass spectrum. A major peak was observed with *m/z* 943 in the non complexed form. In the complexed form *m/z* of 997 was observed which was assumed to be the mass plus ferric iron. The NMR and MS spectrum were analysed with MRenova software and it was matched with its library to divulge a ferrichrome type of siderophore and this did not match with the known siderophores and thus remained unidentified and it considered as a new type of hydroxamate siderophore (Additional file 2: Figs. S4, S5).

3.6. Keratinocyte migration:-

The migratory potential of hydroxamate on keratinocyte was evaluated using standard scratch wound assay. The scratched keratinocyte monolayers were treated with glucotoxic condition with two different concentrations (10 and 30 µg) on hydroxamate siderophore. These concentrations were determined by the result of cell viability test (Fig. 5). In which IC 80 concentrations were 10 and 30 µg of compound and 30 mM glucose condition was taken as glucotoxic condition. The migration rate was thrice in treated cells (Table 5; Fig. 6) when compared to untreated cells indicating that hydroxamate influences keratinocyte cell migration (Additional file 2: Figs. S6, S7, S8).

3.7. Siderophore enriches lamellipodia formation in keratinocyte:-

The lamellipodia formation in keratinocyte was assessed by staining the actin fibers. The phalloidin staining of F-actin bodies showed the lamellipodial points and thick fiber assembly in scratched keratinocytes. The negative control cells showed very few lamellipodia formations when compared to the siderophore treated cells which expressed multiple lamillipodial extensions indicating the enhancement of migratory rate in HaCaT cells by trihydroxamate siderophore (Additional file: Fig. S9).

3.8. Siderophore enhances cell proliferation in keratinocyte:-

The cell proliferation was assessed by MTT assay. The phosphate buffered saline (pH 7.4) was used as positive control because it has potential to heal wound. All the conditions were expressed above 70 % of cell proliferation. The glucotoxic conditions only showed minimal cell proliferation compared to other conditions. The 2 h preincubation of compound suppressed glucotoxicity in keratinocyte. The results of MTT was exposed proliferation potential of trihydroxamate siderophore even in glucotoxic conditions (Fig. 7).

3.9. Reactive oxygen species generation:-

The endogenous generation of ROS was capable to increased keratinocyte migration. It was stimulated to NAD(P)H oxidase (NOX), which is an important source of intracellular ROS. We used the NOX agonist, phorbol myristate acetate (PMA) to stimulate the endogenous ROS in scratched keratinocytes. The addition of PMA enhanced the ROS in the initial minutes, after 5 min the ROS generation was slow down and moderate level was maintained in all the conditions (Fig. 8). Desferol was used as standard treated with Keratinocyte as it expressed elevated production of ROS when compared to other conditions.

3.10. mRNA transcript expressions in scratched HaCat:-

Quantitative PCR analysis revealed that all the twelve genes (SDF, VEGF, FSTL, FGF, MMP9, FOX 1, INTA, INTB, EGF, TGFα, BLT2, and CCL3) were expressed in HaCaT cells (Fig. 9). All the gene expression was observed in 30 μM of siderophore treated cells even in glucotoxic condition. This study revealed that injury-related cytokines, chemokines, and MMP9 were reported to enhance keratinocyte migration.

Discussion:-

4.1. Determination of siderophore production:-

Many fungal organisms are known to secrete siderophores under iron limiting conditions. Siderophore mediated iron transport has been studied in many organisms. However, the mechanism of iron transport, genetic regulation of transport and its medicinal applications are limited. *P.chrysogenum* was found to produce hydroxamate type siderophore under iron deficient conditions. This was determined through both iron perchlorate assay [17] and spectral scan analysis [5]. Growth conditions were optimized to achieve maximum siderophore production.

Three genes, *sid1*, *sid2* and *urbs1* are involved in ferrichrome biosynthesis in *Ustilago maydis* [12]. The *sid1* gene encodes L-ornithine N^5 oxygenase and *sid2* encodes non ribosomal peptide synthetase. This L-Ornithine is an initiator for siderophore biosynthesis pathway, it hydroxylate to form hydroxy ornithine which is first product of this pathway. The basic structural unit of fungal hydroxamate siderophore is N^{δ} –acyl- N^{δ}–hydroxy ornithine [13]. The sid1 plays a major role to catalyse this reaction. *sid2* catalyses the further reaction of acetylated hydroxyornithine covalently binds with glycine to produce ferrichrome type siderophore. Sometimes the hydroxyornithine binds with two amino acids (glycine and serine) to produce deferriferrichrysin. These two genes are expressed in iron rich conditions.

4.2. Assessment of siderophoral activity in *P.chrysogenum:-*

The molecular mass of the siderophore produced by *P.chrysogenum* was obtained by ESI-MS analysis of the purified siderophore obtained from XAD 2 column purified substract obtained from cultures grown under iron-rich conditions. According to the thin layer chromatography results and spectral scan analysis indicated that the siderophore of *P.chrysogenum* is the trihydroxamate as they produced reddish brown spot in the silica gel plate and in the spectral scan the spectrum peak arised from 450–500 nm range [5].

Siderophore is known for its chelating ability to easily adhesion with a keratinocyte cells and chelates the oxidized iron yielded by free radicals via reactive oxygen species (ROS) when the cells get injury. But the ROS play a major role in wound healing. It stimulates wide variety of growth factors and chemokines which are essential to speed up the process of wound healing. But the UC San Diego researchers found that while too much ROS in the cell may be bad, eliminating ROS altogether prevents wound healing. They suggested that the optimal level of ROS signaling is essential for wound healing. In our study, the trihydroxamate was produced the moderate level of ROS in keratinocyte migration. Moreover, the keratinocyte treated with trihydroxamate siderophore manifested an enhanced migration during cell injury. Lamellipodia formation in keratinocyte assured to enhanced cell migration. This lamellipodial protrusions formed by the polymerization of F-actin bodies leading to cell migration. In this study, we revealed that siderophore induced multiple lamellipodial protrusions even in glucotoxic condition to enhance the cell migration, proliferation and it leads to reepithelialization which is a key event in wound healing [6].

This study concludes that trihydroxamate siderophore from *P.chrysogenum* has the potential in the keratinocyte cell migration, cell proliferation, moderate ROS production and to enhance the growth factors and chemokines production. Thus it will be used as therapeutic agent to accelerate wound healing particularly greatly reduced non healing diabetic wound. So we are planning for the Further studies on the expression of all siderophore coding genes in *P.chrysogenum* and evaluating their role in different phases of wound healing may aid in establishing therapeutic targets for impaired wound healing.

Figures:-

Fig 1. Microscopical view of *P.chrysogenum*

Fig 2. Siderophore production on CAS agar plate

Fig 3. Hydroxamate confirmation using iron perchlorate assay

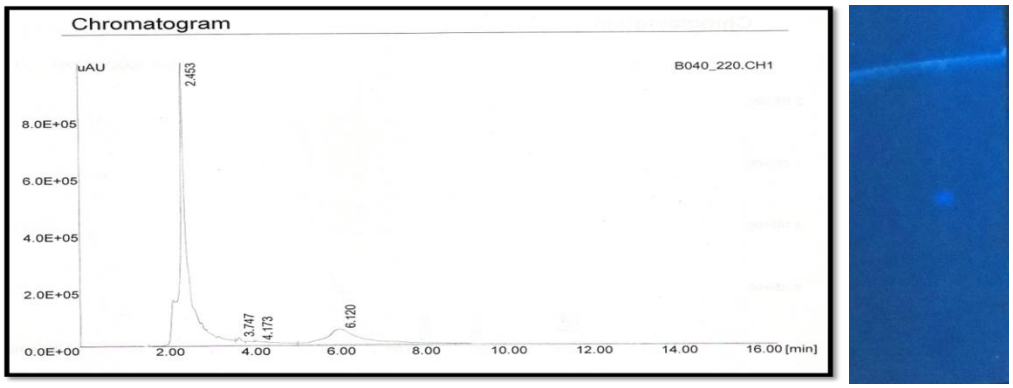


Fig 4a. The purified siderophore eluted from HPLC reverse phase chromatography of sample eluted from XAD 2 resin

Fig 4b. HPLC eluted siderophore compound confirmed on silica gel plate

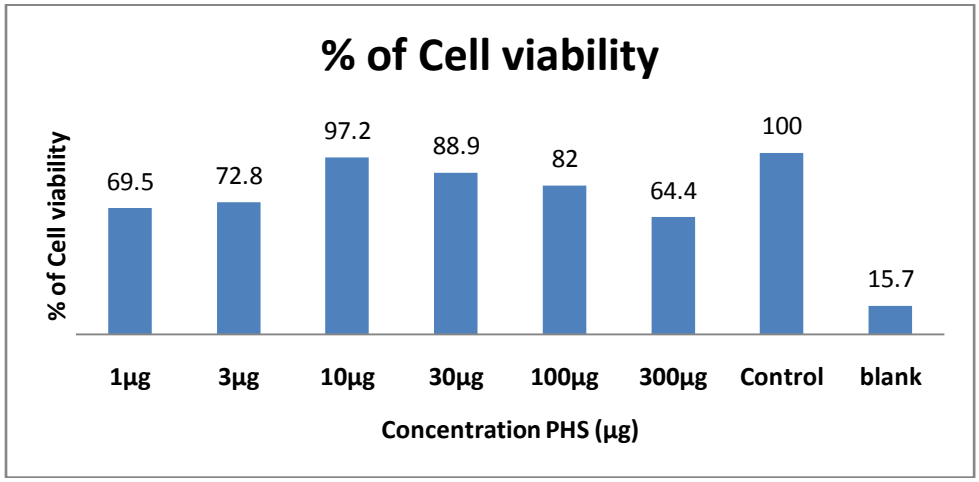


Fig 5. Cytotoxicity assessed for hydroxamates from *P.chrysogenum* on HaCat Cell line

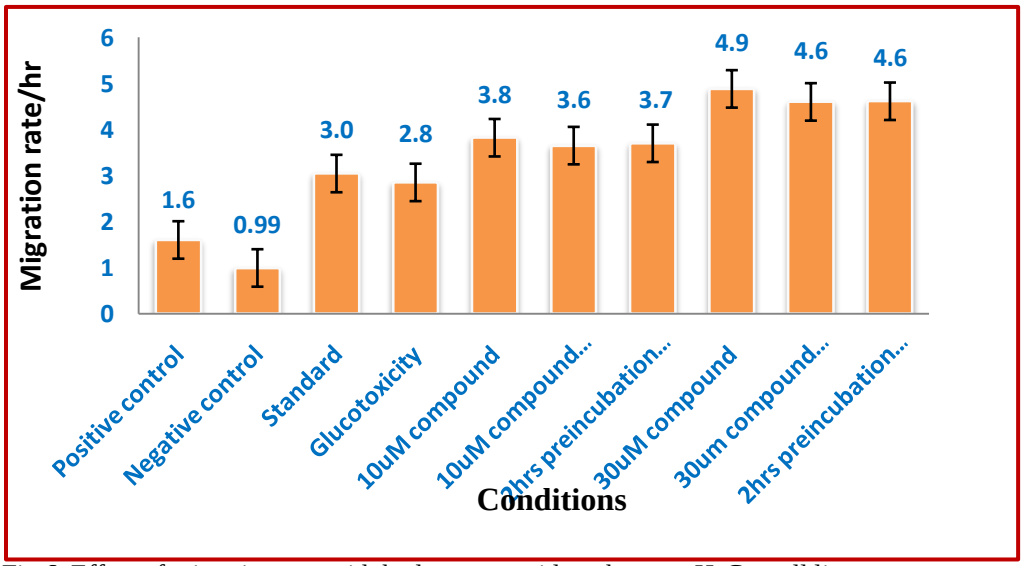


Fig 6. Effect of migration rate with hydroxamate siderophore on HaCat cell line

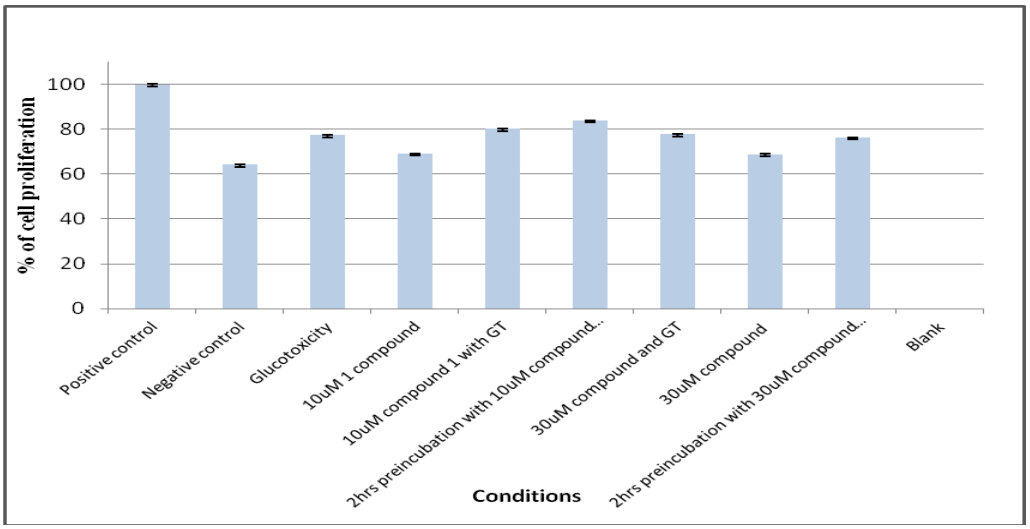


Fig 7. Graphical depiction of the proliferative potential of siderophore on keratinocyte assessed through MTTassay

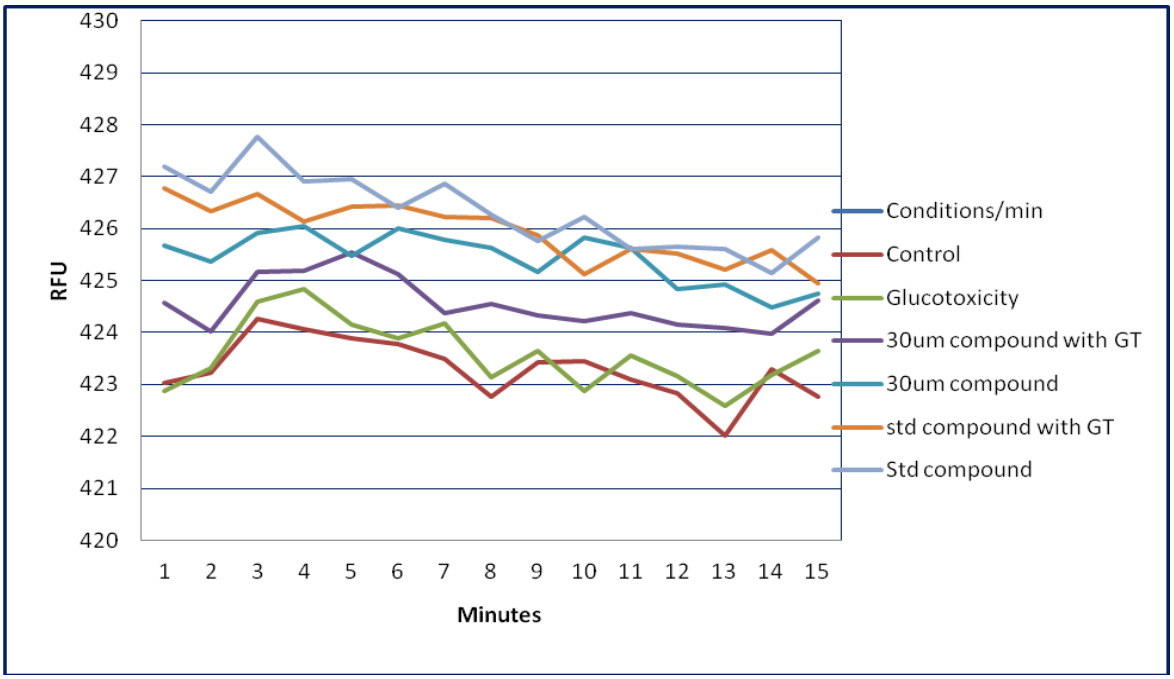


Fig 8. Graphical representation of reactive oxygen species production in cells treated with siderophores and glucotoxic condition

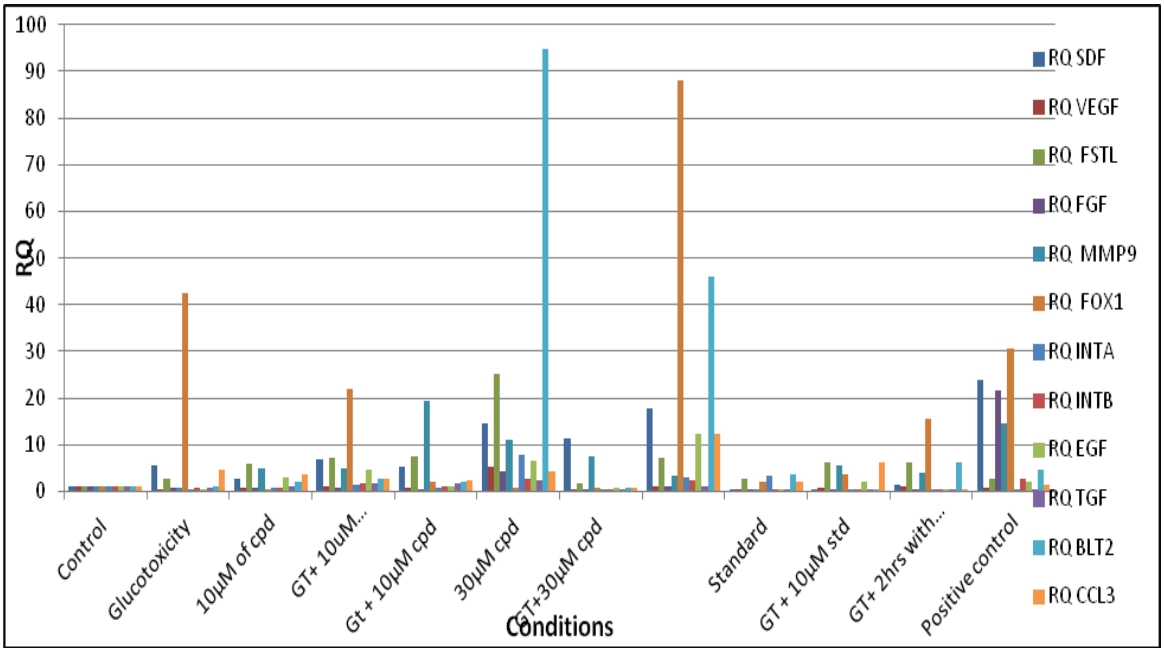


Fig 9. mRNA expression in wounded keratinocyte monolayer cells

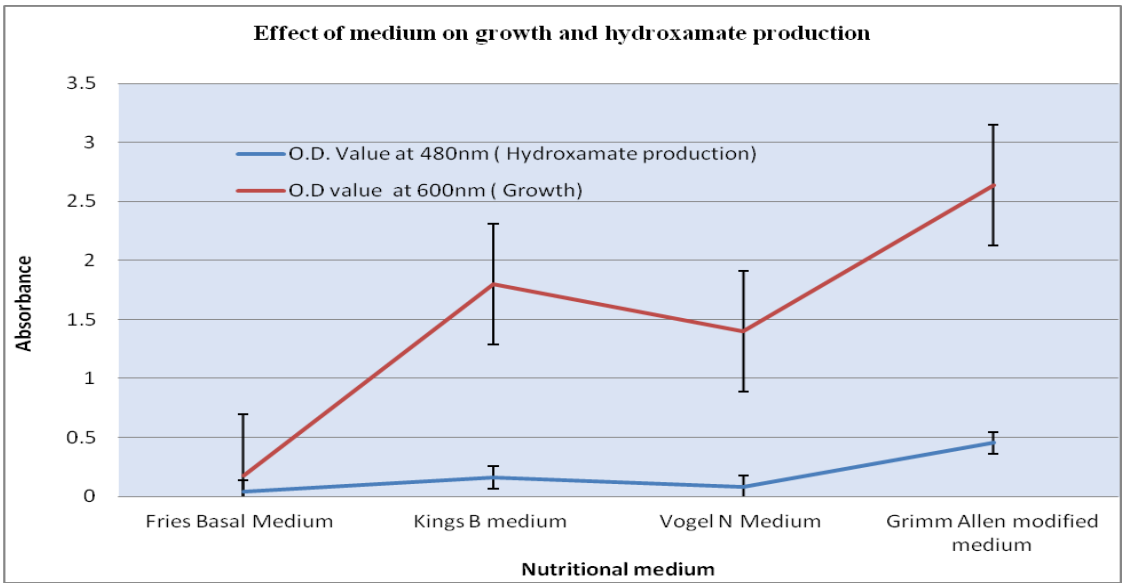


Fig S1. Optimization of medium for *P.chrysogenum* culture growth and hydroxamate production

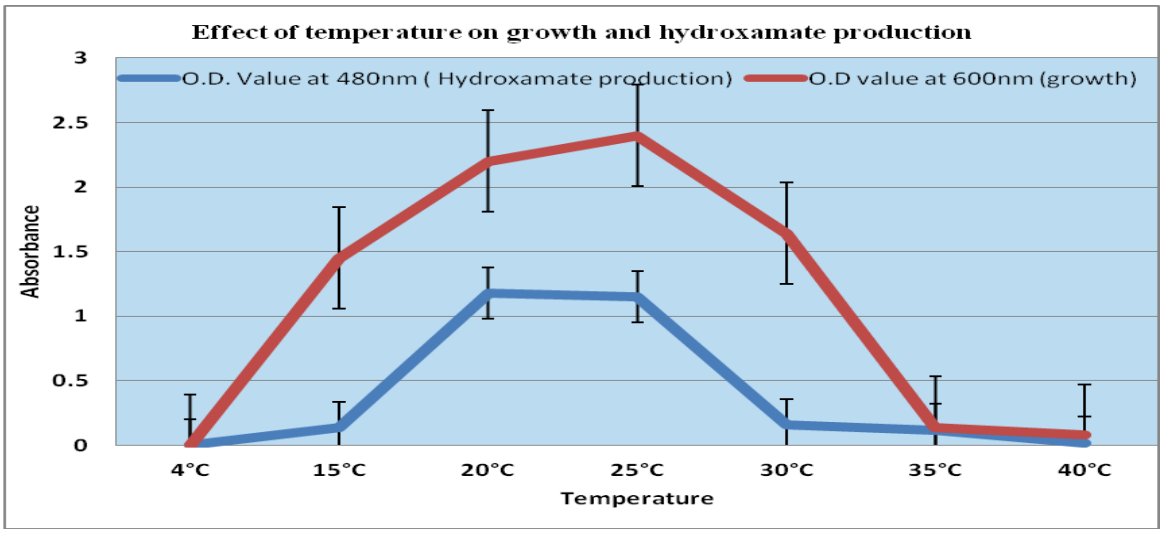


Fig S2. Optimization of temperature for *P.chrysogenum* culture growth and hydroxamate production

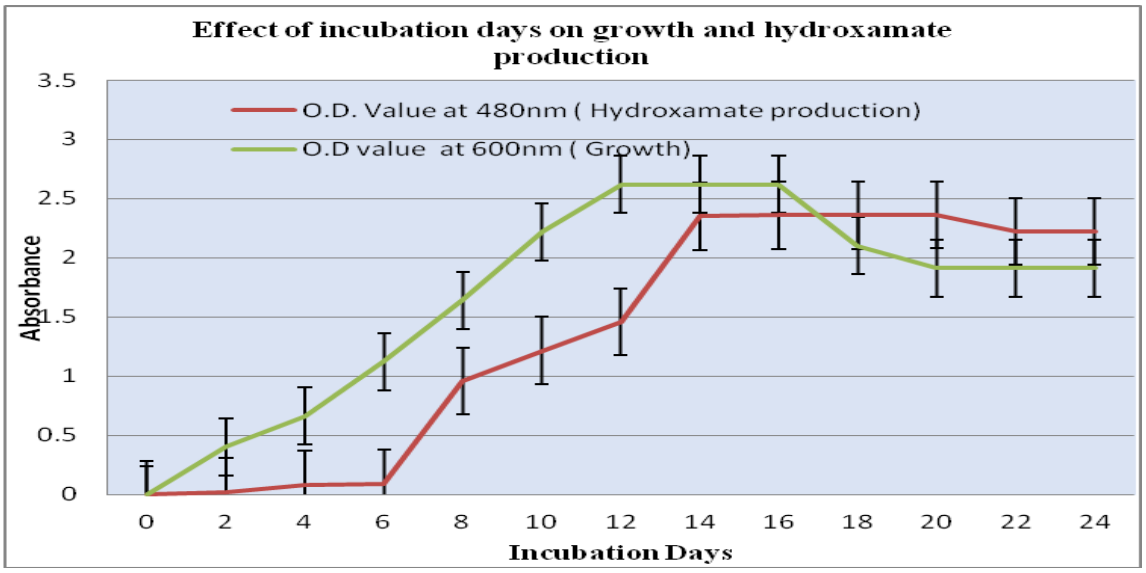


Fig S3. Optimization of incubation days for *P.chrysogenum* culture growth and hydroxamate production

Fig S4: EI MS spectra for trihydroxamate siderophore, the important peaks are indicated with arrows.

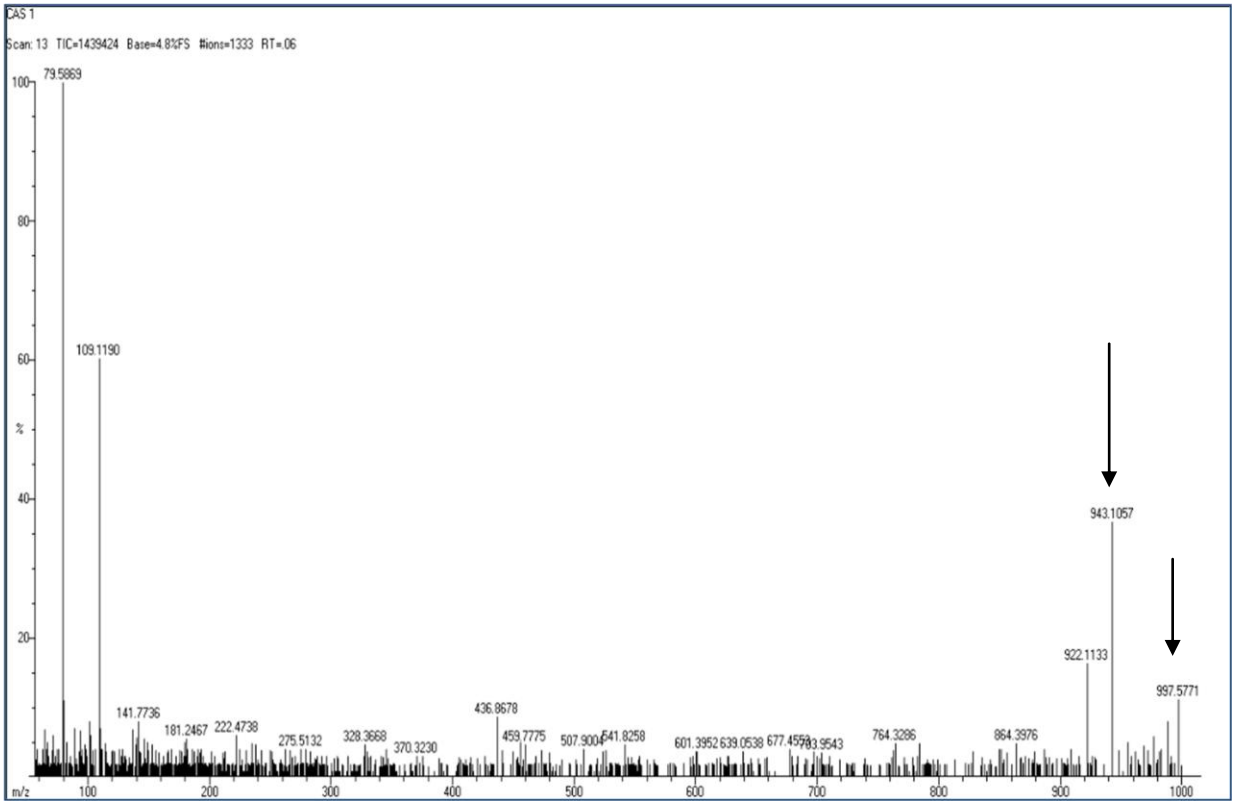


Fig S5. FT-IR prediction of Penicillial hydroxamate siderophore

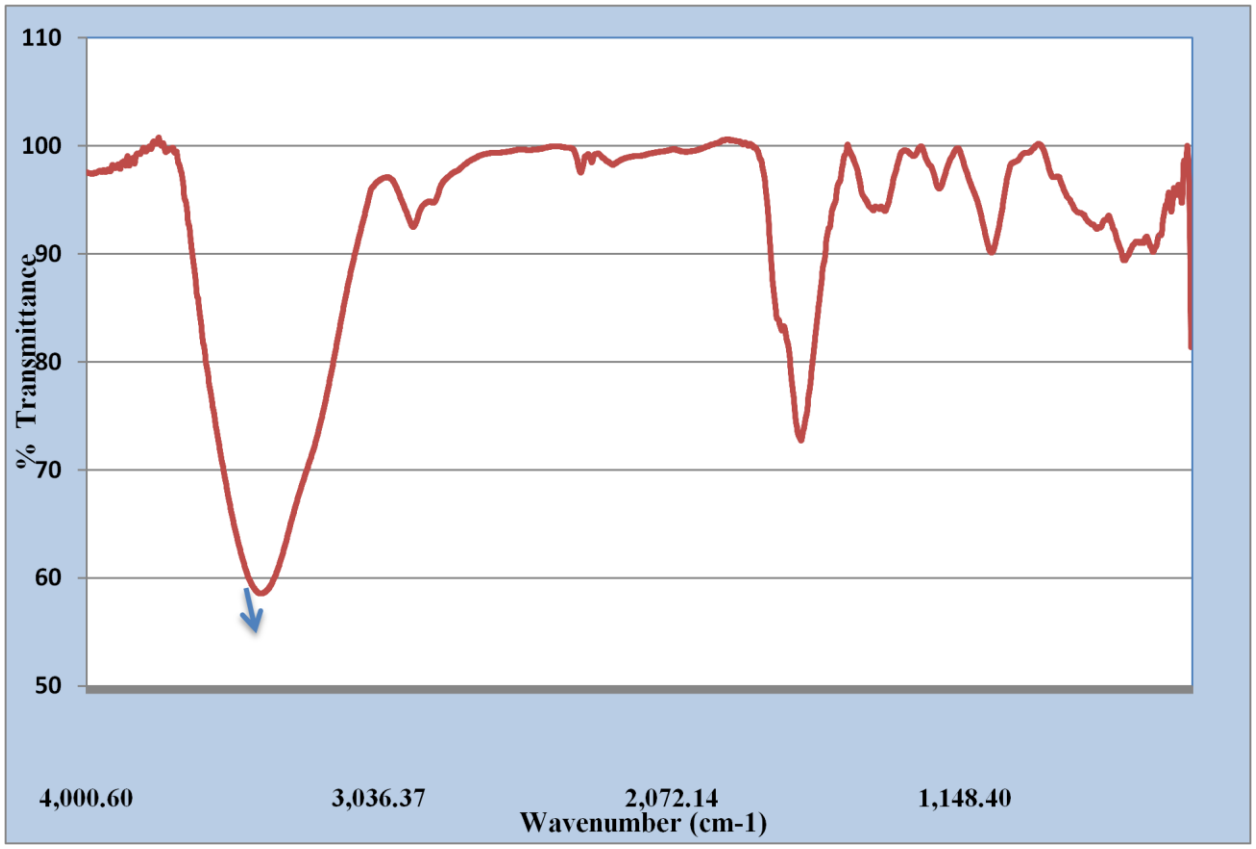
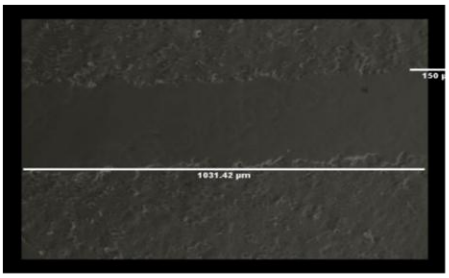
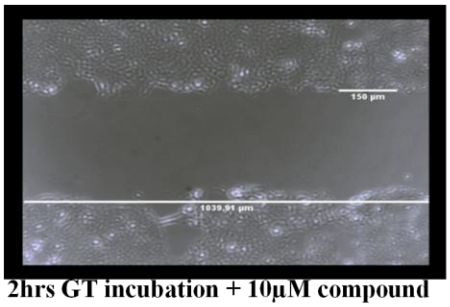


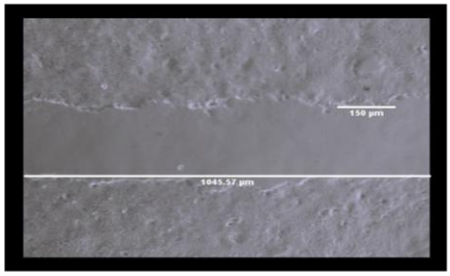
Fig S6. 0 hr cell migration on human keratinocyte (HaCat) cell line



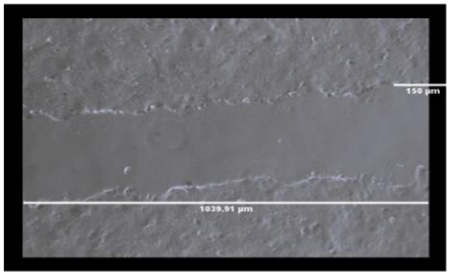
Control



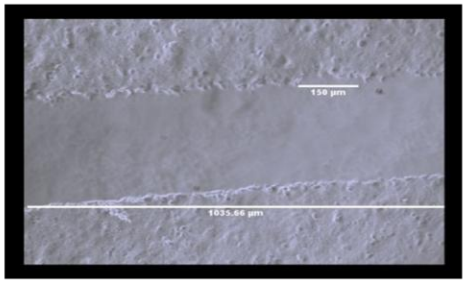
2hrs GT incubation + 10µM compound



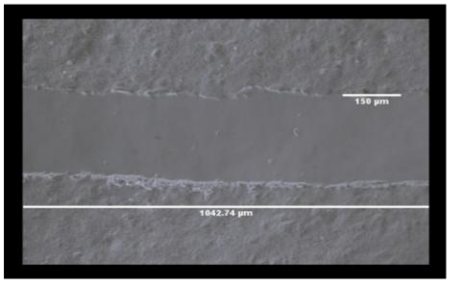
10µM compound



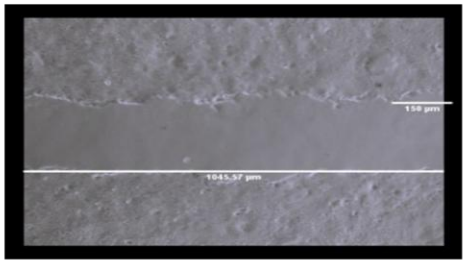
30µM compound



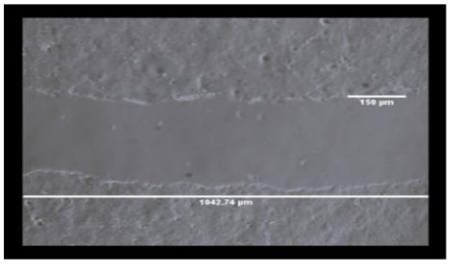
Desferol (+ve control)



Glucotoxic (GT) condition



30µM compound



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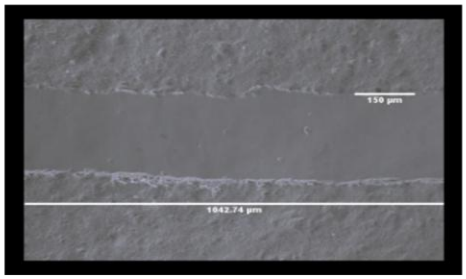
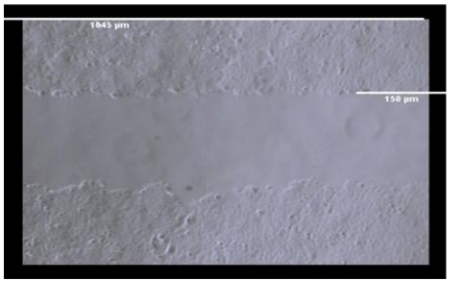
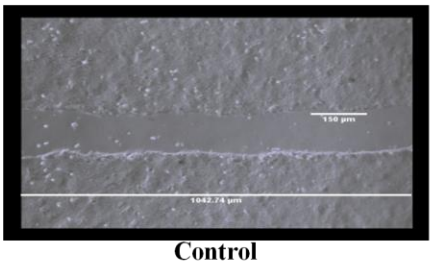


30µM compound

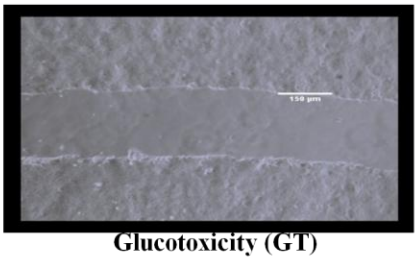


30µM compound

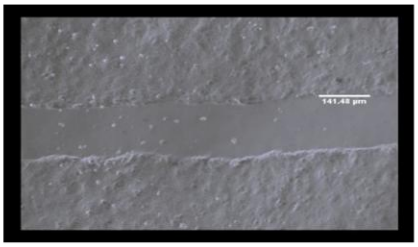
Fig S7. 4 hr cell migration on human keratinocyte (HaCat) cell line



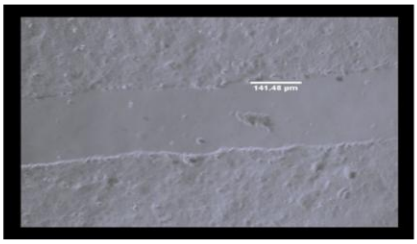
Control



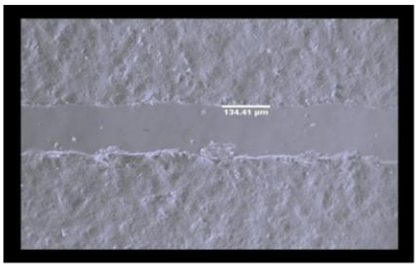
Glucotoxicity (GT)



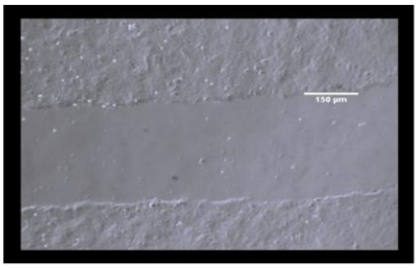
10µM compound



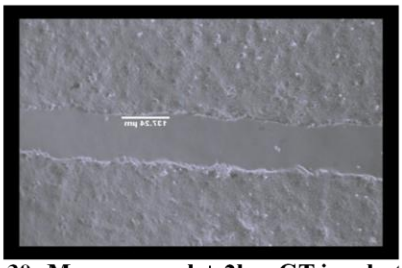
10µM compound + 2hrs GT incubation



30µM compound

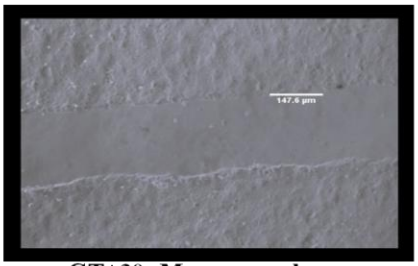


Desferol

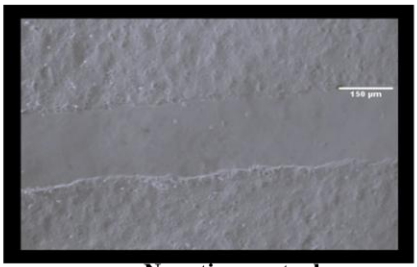


30µM compound + 2hrs GT incubation

GT+10µM compound

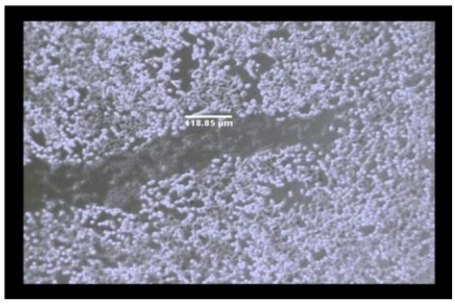


GT+30µM compound

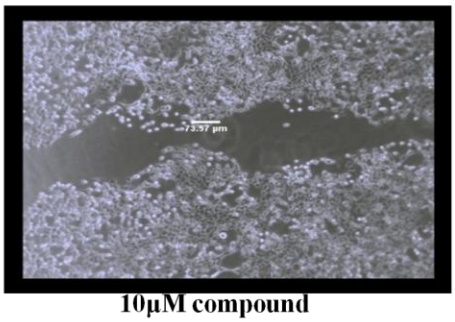


Negative control

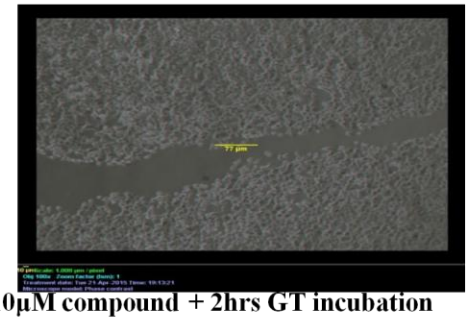
Fig S8. 20 hr cell migration on human keratinocyte (HaCat) cell line



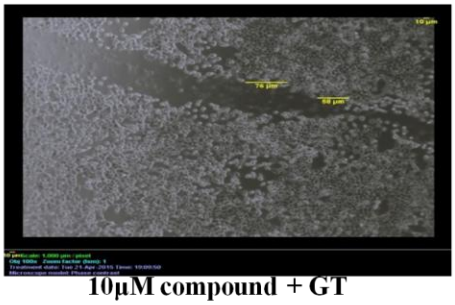
Control



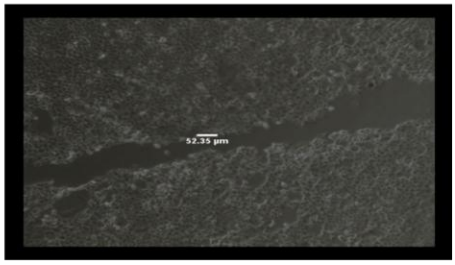
10µM compound



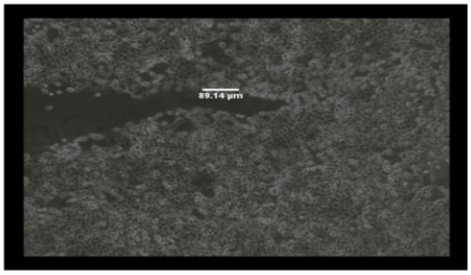
10µM compound + 2hrs GT incubation



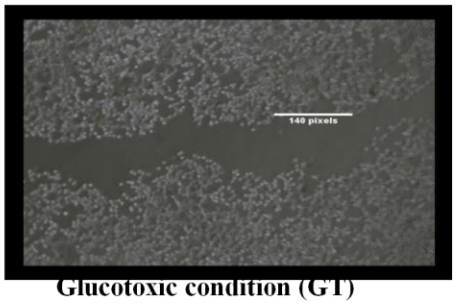
10µM compound + GT



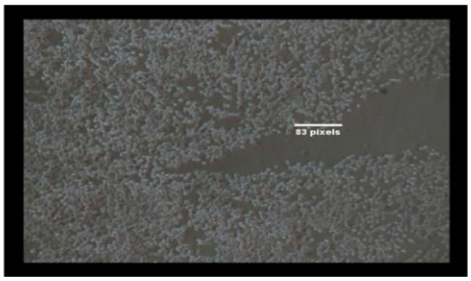
30µM compound



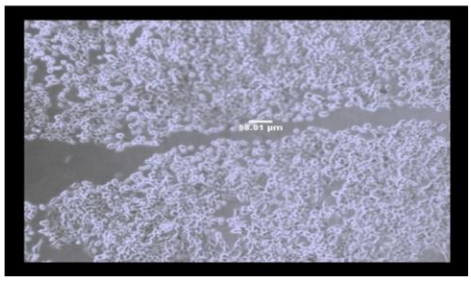
Desferol



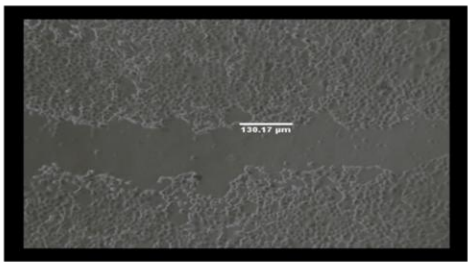
Glucotoxic condition (GT)



30µM compound + 2hrs GT incubation

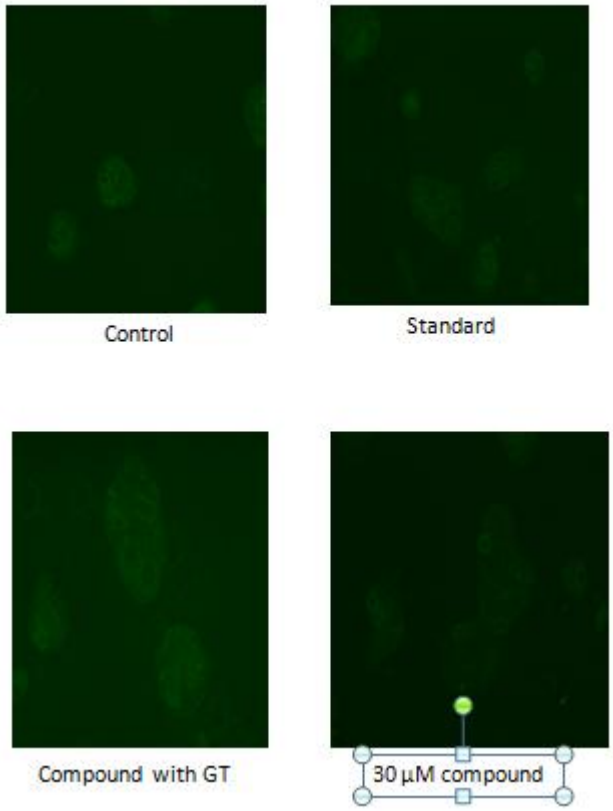


30µM compound + GT



Negative control

Fig 6. Lamidapodial formation on HaCat



Tables:-

Table 1: Estimation of siderophore production using culture filtrate

S.No	Samples	O.D. value at 480 nm	Estimation of siderophore (µg/ml)
1	Control	0.000	0.000
2	Standard	0.095	7.501115
3	*P.Chrysogenum*	0.076	7.425553

Table 2: Detection of Aminoacids in column purified hydroxamate compound

S. No	Solvents	Aminoacids	RF value
1	Acetonitrile: 0.1M ammonium acetate	Tryotophan	0.76
2		L-ornithine	0.8
3		Serine	0.73

Table 3: Spectrum frequency of FTIR

S.No	Band assignment	Frequency (wavenumber, cm^{-1})
1	H–OH stretching	3427.85
2	CH_3	2924.52
3	NH_3^+ stretching	2372.98
4	NH_3^+	2265.95
5	C=O (secondary amide)	1647.88
6	C–H (functional group)	1411.64
7	C–N (NOD bending)	1233.25
8	C–N (NOD bending)	1060.66

Table 4: ^{1}HNMR and ^{13}C NMR spectrum frequency of purified siderophore.

H^1 NMR	**Residue**	**Proton group**
Glycyl	CH_2	3.5826-3.9996 (2H)
	NH	---
Seryl	CH	3.9286-4.0718 (2H)
	CH_2	3.5826-3.7920 (4H)
	OH	5.1152 (2H)
	NH	---
Ornithyl	CH	3.9286-4.3308 (3H)
	CH_2 (β)	1.7241-1.9222 (6H)
	$CH_{2\ (\gamma)}$	1.72 (6H)
	NH	7.2273 (3H)
N-Acyl	CH	6.0876 (2H)
	CH_2	2.30 (4H)/(2H)
	CH_2O	3.58 (4H)/2(H)
	OH	4.33 (2H)/(1H)
	CH_3	1.2659 (3H)/(6H)
^{13}C NMR	**Residue**	**Carbon group**
Glycyl	CH_2 > C=O	43.29 (1C)
Seryl	C-CH_2 –C=O	----
Ornithyl	CH	-----
	CH_2 (β)	28.43
	$CH_{2\ (\gamma)}$	24.5817

Table 5: Effect of hydroxamate siderophore with different conditions on keratinocyte migration.

Conditions	**Total surface area (µm) = b*l**			**Migrated cell surface area=length of cell migration*2*l**			**Percent closure**			**Migration rate (µm/hr)**
	0 h	**4 h**	**20 h**	**0 h**	**4 h**	**20 h (µm)**	**0 h**	**4 h**	**20 h**	
Positive control	154713	156411	123929.6	0	0	9600	0	0	7.74633	1.6
Negative control	156408	156408	135730.9	0	0	5949	0	0	4.382938	0.9915
Standard	155349	155349	92318.73	0	0	18258	0	0	19.77713	3.043
Glucotoxicity	156835.5	156835.5	97238.01	0	0	17100	0	0	17.58572	2.85
10 µM compound	156835.5	147927.2	76922.58	0	2556	22929	0	1.727876	29.80789	3.8215
10 µM compound with GT	156835.5	147927.2	79463.32	0	2556	22200	0	1.727876	27.93742	3.65
2 h preincubation with 10uM compound and GT	156835.5	138015.2	80508.89	0	5400	21900	0	3.912611	27.20196	3.7
30 µM compound	156411	140535.1	54735.59	0	4677	29295	0	3.327995	53.52094	4.8825
30 µm compound with GT	156406.5	153698.8	60653.52	0	900	27597	0	0.585561	45.49942	4.5995
2 h preincubation with 30uM compound and GT	156411	143682.2	76326.61	0	3774	27681	0	2.62663	36.26651	4.6135

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Authors' contributions:-
SKK designed and performed the experiments and prepared the manuscript. SKK and SR equally performed the cell culture work. CSK contributed ROS experiment and gene expression studies. VK approved the experiments and corrected the manuscript and MB provided the permission to carryout cell line experiments and corrected the experimental designs. All authors read and approved the final manuscript.

Competing interests:-
The authors declare that they have no competing interests.

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