Biological Evaluation and Computational Studies of Methoxy-flavones from Newly Isolated Radioresistant *Micromonospora Aurantiaca* Strain TMC-15

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Abstract

This study aims to determine UV-B resistance and to investigate computational analysis and antioxidant potential of methoxy-flavones of *Micromonospora aurantiaca* TMC-15 isolated from Thal Desert, Pakistan. The cellular extract was purified through solid phase extraction and UV-Vis spectrum analysis indicated absorption peaks at $\lambda_{\max}$ 250 nm, 343 nm, and 380 nm that revealed the presence of methoxy-flavones named eupatilin and 5-hydroxyauranetin. The flavones were evaluated for their antioxidant as well as protein and lipid peroxidation inhibition potential using di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH), 2,4-dinitrophenyl hydrazine (DNPH), and thiobarbituric acid reactive substances (TBARS) assays, respectively. The methoxy-flavones were further studied for their docking affinity and interaction dynamics to determine their structural and energetic properties at atomic level. The antioxidant potential, protein and lipid oxidation inhibition and DNA damage preventive abilities were correlated as predicted by computational analysis. The eupatilin and 5-hydroxyauranetin binding potential to their targeted proteins 1N8Q and 1OG5 is -4.1 and -7.5 kcal/mol, respectively. Moreover, the eupatiline and 5-hydroxyauranetin complexes illustrate van der Waals contacts and strong hydrogen bonds to their respective enzymes target. Both *in-vitro* studies and computational analysis results revealed that methoxy-flavones of *Micromonospora aurantiaca* TMC-15 can be used against radiation mediated oxidative damages due to its kosmotrophic nature. The demonstration of good antioxidant activities not only protect DNA but also protein and lipid oxidation and therefore could be a good candidate in radio-protective drugs and as sunscreen due to its kosmotropic nature.

Introduction

Low-wavelength radiation is highly mutagenic and can damage all living organisms. Some radioresistant extremophiles have adapted themselves to thrive in the presence of high levels of radiation by effective scavenging abilities of reactive oxygen and nitrogen species (Fredrickson et al., 2008). Microorganisms residing in such extreme habitats can evolve faster as compare to those living in benign environment (Li and Hong, 2016). This is associated to the high mutation rate in such harsh conditions which ultimately could lead to put selective pressure on these microbes as a result, produce something interesting of biotechnological use (Sayed et al., 2020). Diverse metabolic properties in radioresistant organisms include production of extremozymes, carotenoids, flavonoids, vitamins and other potential extremolytes that can scavenge reactive radicals (Yu et al., 2015). Radioresistant extremophiles act as host for novel compounds that are not only important for their structural and biochemical diversity but also devise biotechnological applications in food, cosmetics and pharmaceutical industries. Such metabolic products not only aid in the survival of microorganisms in extreme environment but also play a role in conditions like desiccation resistance, involved in immunosuppression, enhancing antioxidant and anticancer properties, enabling oxidative damage repair (Nair et al., 2004). The exploration of radioresistant extremophiles has helped to develop bio-products for novel and improved drugs that offer protection to lipids, proteins, and nucleic acids (Waditee-Sirisattha et al., 2016). The demand of

radioresistant, protective biomolecules has increased in the pharmaceutical and biotechnological industries (Galano et al., 2010).

Various extremolytes have been extracted from radioresistant bacteria in search for radioprotective biomolecules. Flavonoid metabolites are promising as effective antioxidant candidates (Lee et al., 2009) as they can interfere with signal transduction pathways and limit proliferation and metastasis. Flavonoids counter with reactive radicals and produce stabilized and inactive radicals (Hanasaki et al., 1994). Flavonoids can also directly scavenge free radicals. To date, there is limited information available on bioactive compounds from radiation resistant bacteria although radioresistant bacteria are ubiquitous in the environment (Beblo-Vranesevic 2017). Some of the extremophiles are also found in the genus *Micromonospora*, belongs to Actinobacteria. These actinobacteria have biosynthetic gene clusters, which encode various biosynthetic pathways of potentially useful metabolites (Li and Hong, 2016). Extreme environments have vast diversity of actinobacteria that could be the source of radiation protective metabolites. Actinobacteria isolated from hot spring has also been recently evaluated for bioactivities which exhibited strong antibacterial activity against many pathogenic microbes (Mehetre et al., 2019). Some studies showed that the cell wall of *Micromonospora* isolated from high altitude Atacama Desert soil contains *meso*- and *hydroxy*-diaminopimelic acid, and glycolipids (Carro et al., 2019). A recent study conducted on microbial colonization process on solar panel surfaces and its effect on photovoltaic efficiency also revealed the presence of extremophilic bacterial genera *Deinococcus*, *Hymenobacter* and *Roseomonas* and fungal *Neocatenulostroma* (Tanner et al., 2020).

In the present study, a radiation-resistant bacterium was isolated from a soil sample of the Thal Desert. Subsequently, the growth conditions and *in-vitro* biological activities of flavones were characterized for this radioresistant isolate. The intracellular methanol-soluble compounds from the isolated strain was extracted and purified for characterization and further bioactivities. The radiation-protective properties of the metabolites were screened with a view to potential biotechnological applications with emphasis on nutraceutical industry.

Materials And Methods

Isolation of UV-B radiation resistant strains

Soil samples were collected from the Thal Desert Pakistan, diluted serially and plated on tryptone glucose yeast extract agar (TGY) containing (per liter) 5 g tryptone, 1 g glucose, 5 g yeast extract, and 1 g K₂HPO₄, pH 7.0. Before incubation, the plates were exposed to UV radiation for 5 min in a UV-B chamber (119× 69× 52 cm) supplied with 280 nm UV light of 20W. The rate of UV fluency (energy/area/time (J/m²)) for test samples was calculated by using the formula $H_e = E_e \times t$ where H_e is radiant exposure, E_e is energy of irradiance and t is the time duration maintained during radiation exposure (Sajjad et al., 2017). The survival curves were determined by sub-culturing the bacteria from the radiated plates.

Bacterial survival under UV-B radiation and oxidative stress

The resistance of strain TMC-15 to UV-B radiation, hydrogen peroxide and mitomycin C was tested by plotting the survival curve as described previously by Mattimore and Battista (1996). The cell culture was serially diluted (1:000) optical density (OD₆₀₀) of 0.08-0.1 (McFarland standard) in sterile phosphate buffer solution (PBS) pH 7.0 and spread on TGY agar plates, followed by exposure to various doses of UV-B radiations at 280 nm and incubation for 3 to 7 days at 35°C. The survival was estimated by dividing total number of colonies on radiated plates to colonies on non-radiated (control) plates. For oxidative stress determination, strain TMC-15 (OD₆₀₀ of 0.08-0.1) was treated with 10-60 mM H₂O₂ and 2-10 µg/ml mitomycin-C for 30 min, followed by plating on TGY and incubation at 35°C for 3 to 7 days. The relative survival was calculated as the difference in the number of colonies between the treated and untreated cultures. All experiments for the survival curve were run in triplicate.

Bacterial identification

Molecular identification was carried out by extracting DNA, using a modified SDS based DNA extraction method (Xia et al., 2019). 16S rRNA gene sequences were then amplified with universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR amplicons were sequenced at Macrogen Service Center (GeunChun-gu, Seoul, South Korea) and computed by using basic local alignment search tool (BLAST) and compared with homologous sequences in databases of NCBI for nearest relatives. A neighbor-joining tree was constructed using MEGA X (Kumar et al., 2018) for identification of strain TMC-15 and compared with already reported strains in NCBI GenBank for acquisition of accession number. The 16S rRNA sequence of strain TMC-15 was submitted to NCBI GenBank under the accession number MN721337.

Extraction of intracellular bioactive compounds from strain TMC-15

Strain TMC-15 was grown under optimum conditions with continuous shaking at 150 rpm. The cells were harvested by centrifugation (5000 × *g*) for 15 min at 4°C, and the cell pellets were washed using PBS (pH 7.4) before placed at -4 °C for cold shock. Methanol was added to the pellets and disrupted repeatedly by sonication until the suspension became yellow colored. The suspension was centrifuged, then passed through syringe filter (0.22 µm pore size) and finally dried out in order to remove the solvent.

Characterization of bioactive compounds from strain TMC-15

Fourier transform Infra-Red (FT-IR) analysis

The methanolic extract was vacuum evaporated to collect the sticky material (yellow pigment) and was further subjected to drying at 30°C, the compound once purified using SPE C18 cartridge was subjected to FTIR analysis. The purified extract (10 mg sample) was mixed with 100 mg KBr for Fourier Transform Infra-Red (FTIR) analysis (Jiao et al., 2010). Absorption measurement was taken over the wavelength range of 400 to 4500 cm⁻¹ and the spectra were recorded using a Spectrum 65 FTIR spectrometer

(PerkinElmer). The extract was re-dissolved in methanol and the spectrum in the range of 200-800 nm was recorded with UV-Vis spectrophotometry.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The purified extract (solid phase extraction) was analyzed with an Agilent 6310 Ion Trap LC-MS. The extract was dissolved in methanol (LC-MS grade) and injected to column (C18, internal diameter 5 μm , 150 $\times$ 4.6 mm). Mobile phase was in isocratic mode consisting of acetonitrile: water (80:20) with 0.5 ml/min flow rate. The MS was used in negative ion mode to detect mass to charge (m/z) transitions $[M+H]^-$. The data was resolved with Thermo Scientific XcaliburTM software.

Bioassays of purified extract from strain TMC-15

Antioxidant activity by Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The DPPH scavenging rate of the purified intracellular extract was measured by the method as previously described (Ebrahimzadeh et al., 2008). The reaction mixture contained 20 μl DPPH (0.1 mM) and 180 μl of the sample at different concentration (10-50 $\mu\text{g } \mu\text{l}^{-1}$). The assay mixture (200 μl) was incubated in dark for 30 min at 30 $^{\circ}\text{C}$ before the absorbance was measured at 517 nm. Ascorbic acid (10-50 $\mu\text{g } \mu\text{l}^{-1}$) was used as a positive control. The relative scavenging activity was calculated using the formula $[(\text{Control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100$.

Iron chelation assay

Iron chelation activity was determined by adding 50 μl of 2 mM ferrous chloride to 10-50 $\mu\text{g } \mu\text{l}^{-1}$ of the purified sample (Zhao et al., 2008). The reaction was initiated by adding 20 μl of ferrozine (5 mM). The assay mixture was incubated at room temperature for 15 min in dark and the absorbance at 562 nm was measured. EDTA (10-50 $\mu\text{g } \mu\text{l}^{-1}$) was a positive control and a negative control was run in parallel without any chelating agent.

Protein carbonylation assay

100 μl of bovine serum albumin (BSA) was incubated with 10-50 $\mu\text{g } \mu\text{l}^{-1}$ of the sample (1:1). The assay mixture received 50 μl FeSO_4 (1 mM), 50 μl of H_2O_2 (80 mM). The mixture was incubated for 1 h at room temperature and the reaction was stopped by adding 2-3 drops of catalase. This mixture was incubated with 300 μl 2, 4-dinitrophenylhydrazine (DNPH) (10 mM) for 1 h in dark and vortexed after every 10 min. The unbound protein was precipitated by adding 50 μl of chilled 10% trichloroacetic acid (TCA). Protein precipitates were washed with 50% ethyl acetate and ethanol (1:1) (Sobeh et al., 2019). TCA was evaporated and precipitates were centrifuged at 2000 $\times g$ at 4 $^{\circ}\text{C}$ for 10 min after adding 1 ml of guanidine hydrochloride (6 M). Absorbance of the supernatant was measured at 370 nm compared to a

blank run in parallel by replacing H_2O_2 and FeSO_4 with distill water. Percentage inhibition was calculated from $[(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}] \times 100$.

Lipid peroxidation inhibition assay

Thiobarbituric acid assay was performed by measuring lipid peroxidation inhibition offered by flavones. 1 g rat liver tissue was fused (Bio-Gen PRO200 homogenizer) in Tris-HCl buffer at pH 7.4 and centrifuged at $3000 \times g$ for 5 min. An aliquot (1 ml) was mixed with $10\text{--}50 \mu\text{g} \mu\text{l}^{-1}$ of the sample. The reaction was initiated by mixing 300 μl of FeSO_4 (10 Mm), 200 μl sodium dodecyl sulphate (SDS) (8.1%), 250 μl acetic acid, and 250 μl thiobarbituric acid (TBA) (0.6% v/v). The assay mixture was incubated for 1 h at 100 °C before the absorbance was measured at 532 nm. Finally, the lipid peroxidation inhibition was calculated using malondialdehyde (MDA) standard curve.

DNA protection activity

The DNA damage prevention assay was determined by incubating pUC18 plasmid (2 μl) treated with $10 \mu\text{g} \mu\text{l}^{-1}$ of purified sample, 3 μl of FeSO_4 (2mM), 4 μl of H_2O_2 (30%), 4 μl of sodium nitroprusside (1 mM) followed by incubation for 1 h at 37°C (Kitts et al., 2000). Double and single stranded breakage of pUC18 was generated by the attack of hydroxyl radical, observed in the negative control. In the positive control, untreated DNA was run in parallel prior to addition of any DNA damaging agents. The DNA bands of the treated extract, the negative and positive controls were separated using agarose gel electrophoresis.

Computational Studies

Molecular Docking

Molecular docking of the compounds with representative two enzymes that respond to production of reactive oxygen species was accomplished using Autodock 4.2.6 (Morris et al., 2009). Initially, the enzymes 3D structure of the cytochrome P450 (PDB ID: 1OG5) and lipoxygenase (PDB ID: 1N8Q) were retrieved from protein data bank (Bermen *et al.*, 2000) and subsequently treated in a short receptor preparation phase. During this phase, all co-crystallized ligands were removed from the receptors and hydrogen atoms were added. Energy minimization was performed to remove steric clashes in the enzymes structure limiting steepest descent steps to 100, conjugate gradient steps to 10 and setting the step size to 0.02 Å. AMBER ff14SB force field was applied on the structure to treat standard residues whereas to specify net charge for the receptors AM1-BCC charge method was used. The docking procedure used in this study following protocol described by Costa et al., (2018). Before, the docking method was validated by docking the co-crystallized ligand at the same position presented in the crystal structure. For both compounds, 10 iterations were produced and ranked as per binding affinity for the receptors (binding energy in kcal/mol). Coordinates of the grid center for 1N8Q on XYZ were set as: 21.864 Å, 2.184 Å and 18.909 Å whereas for 1OG5 it is -20.257 Å, 86991 Å, and 38.581 Å. The grid six points for 1N8Q were 24X, 18Y and 12Z, while for 1OG5 they were 22X, 20Y and 24 Z. Conformation with high affinity (lowest binding energy) among the predicted poses was selected as complex with the enzyme to be visualized for interactions and put forward possible interaction mechanism.

Molecular Dynamics (MD) simulations

The selection of screened small compounds for potential optimization using MD simulations depends not only on the docking energies but also on interacting residues, fitness scores, pharmacophoric and low toxicity features. The study of conformational behavior of selected compounds in complex with macromolecules at atomic level is an important step in providing an insight into the structural stability, physico-chemical behavior, and biological mechanisms. In view of this, we performed a 50ns MD simulation for the prioritized complexes using AMBER 18. Using antechamber, a module embedded in Amber18, a set of initial parameters and libraries was generated (Case *et al.*, 2008). In tleap module, ff14SB force field for proteins and GAFF for ligands were used to investigate the stability and dynamical properties of selected compounds and dock complexes. In orthorhombic TIP3P water box at 8.0 Å, the top scoring docked conformations of all the four systems were solvated. After that, the Na⁺ ions were added to neutralize the systems. Energy optimization was accomplished via 2000 steps minimization of hydrogen atoms, 1000 steps minimization of system solvation box energy with the restraint of 200 kcal/mol – Å² on the rest of the system, 1000 steps minimization of the entire set of system atoms with the restraint of 5 kcal/mol – Å² exercised on system carbon alpha atoms, and 300 steps minimization on non-heavy atoms of the system with the restraint of 100 kcal/mol – Å² on the remaining components of the system. The system was then heated to 300K through the NVT ensemble supported via Langevin dynamics and SHAKE algorithm to limit hydrogen bonds. An equilibration for 100-ps was attained while sustaining pressure on the system via NPT ensemble letting restraint on Cα atoms of 5 kcal/mol – Å² (Abbasi et al., 2016; Naz et al., 2020). The resulting trajectories, using the CPPTRAJ module, were examined, for the structural stability (Roe and Cheatham 2013).

MMPB/GBSA analysis for binding free energies

The MMPB/GBSA method approach was further applied on the simulation trajectories to estimate free binding energy of all the four system as a difference between the receptor proteins, ligands and receptor-ligand complexes free energies (Miller et al., 2012). The MMPB/GBSA free binding energy is computed using equation:

$$\Delta G_{\text{bind}} = G_{\text{receptor-ligand}} - (G_{\text{receptor}} + G_{\text{ligand}})$$

For each term, the free energy is decomposed into MM energy, polar and nonpolar solvation energy and entropy, as demonstrated by the following equation:

$$\Delta G = \Delta E_{\text{molecular mechanics}} + \Delta G_{\text{solvation}} - T \cdot \Delta S = \Delta E_{\text{sum of angles}} + \Delta E_{\text{van der Waals}} + \Delta E_{\text{electrostatic}} + \Delta G_{\text{solvation, polar}} + \Delta G_{\text{solvation, non-polar}} - T \cdot \Delta S$$

The delta MMPB/GBSA energy of the compounds is calculated from 100 snapshots extracted from the simulation trajectories.

Statistical analysis

Survivability of strain TMC-15 isolated from desert soil following exposure to different energy levels of ultraviolet B (UV-B) radiation. a UV radiation-resistant potential of WMA-LM9, b resistance to different concentrations of hydrogen peroxide (H_2O_2 ; mM), c resistance to different concentrations of mitomycin C ($\mu\text{g/ml}$). Percentage survivability value is calculated as $N_1/N_0 \times 100$ where N_i is the value after exposure to irradiation, H_2O_2 or mitomycin C, and N_0 is the value at time 0, for each condition tested. Results are highly significant among different groups at $p < 0.05$ (Student's unpaired t test). Values are given as the mean $\pm$ standard deviation (SD; error bars) of triplicate experiments. Percentage scavenging activity of flavones were studied by regression analysis between the ability to quench the superoxides and their respective concentrations. Single factor and two-way analysis of variance were applied for analysis of DPPH, iron chelation, lipid and protein oxidation inhibition assays. All the experiments were run in triplicates ($n=3$). The vertical error bars represent standard deviation values ($\pm$ S.D.).

Results

Sample collection and screening of UVR microbes

The results of bacteriological analysis of sand samples collected during summer months have shown 18 UV-resistant isolates from Thal desert. Majority of them were chromogenic where isolate TMC-15 showed high resistant to UV rays and was also able to produce pigments on the surface of TGY agar plates.

Oxidative stress resistance in TMC-15

Strain TMC-15 exhibited 56% survival at $4.068 \times 10^3 \text{ J/m}^2$ of UV radiation (280 nm) (Fig. 1a), 53% survival in the oxidative stress test with 40 mM of H_2O_2 (Fig. 1b), and 52% survival at 6 $\mu\text{g/ml}$ of mitomycin-C (Fig. 1). *E. coli* was run as control which unable to sustain on high dose of UV radiation and high concentration of H_2O_2 , mitomycin C. survival of Strain TMC-15 gradually decreased with increase in concentration of all the three variables. Results are expressed as means $\pm$ SD and were compared using the Student's unpaired t-test. Moreover, the percentage values had an exponential distribution. Error bars represent standard deviation for triplicate experiments. $P < 0.05$ is considered significant.

Identification of strain TMC-15

Gram staining revealed that isolate TMC-15 is Gram positive. Biochemical characteristics revealed that strain is catalase positive, with pale orange, pointed, small colonies on TGY agar plates. 16S rRNA gene sequencing of TMC-15 demonstrated closest alignment to the phylum actinobacteria, genus *Micromonospora*. A phylogenetic tree constructed by Neighbor-Joining method presented 100% match of strain TMC-15 with *Micromonospora aurantiaca* MH588238 (Fig. 2).

Characterization of cellular extract from *M. aurantiaca* strain TMC-15

FT-IR analysis

The FTIR spectra (Fig. 3) revealed peaks at 3258 cm^{-1} representing OH group of alcohols and peaks in the range 2921 and 2851 cm^{-1} representing C-H groups of alkanes. The peak at 1736 cm^{-1} represented the presence of aromatic compounds and the 1223 and 1066 cm^{-1} peaks represent ether linkages (Table 2). The purified intracellular extract was scanned between 200 to 800 nm . The absorption spectra λ_{max} for the purified fraction dissolved in methanol was noted at 343 nm , 250 nm and 380 nm .

Table 1
Antioxidant activities of intracellular compound extracted in comparison to their respective standard (control)

Concentration in $\mu\text{g ml}^{-1}$	10	20	30	40	50
DPPH Radical Scavenging Assay					
% inhibition of Test (Intracellular extract)	70.43	72.2	74	87.3	96.1
% inhibition of Control (Ascorbic acid)	80.2	88	91.2	95.3	97.61
Iron chelation Assay					
% inhibition of Test (Intracellular extract)	34	61	66	76	87.3
% inhibition of Control (EDTA)	65.4	72.68	76.5	89	92.9
Protein oxidation Inhibition Assay					
% protein oxidation inhibition of Control (Ascorbic acid) on BSA	70.34	72.85	74.55	87.3	96.1
% protein oxidation inhibition of Control (Ascorbic acid) on mice liver	77	79.1	79.1	81.2	84
% inhibition of Control (Ascorbic acid)	80	88	91.2	95.33	97.61
Lipid Per-oxidation Inhibition Assay					
% Lipid per-oxidation inhibition of Test (Intracellular extract)	25.5	33.2	42.3	55	70.2
% Lipid per-oxidation inhibition of Control (Ascorbic acid)	44	56	60.2	70	75.1

Table 2
Wave number recorded, type of bond present and functional group representation in FTIR analysis.

Wave Number (cm ⁻¹)	Bond	Functional Group
3258	O-H	Alcohol
2921	C-H	Alkane
2851	C-H	Alkane
1736	C-H	Aromatic Compound
1633	C=C	Alkene
1466	C-H	Alkane
1403	O-H	Alcohol, Carboxylic
1223	C-O	Ether
1066	C-O	Ether

LC-MS analysis

The methanol-soluble extract exhibited a pool of peaks at a retention time of 2.8 min, with two major peaks at highest intensity (Fig. 4). The mass scan width in the negative ion mode was 100–1000. Major peaks for m/z 343.9 and 386.9 matched the formula C₁₈H₁₆O₇ and C₂₀H₂₀O₈, corresponding eupatilin (5,7-dihydroxy-3', 4', 6-trimethoxyflavone) and hydroxyauranetin (5-hydroxy-3,6,7,8,4'-pentamethoxyflavone).

Bioassays of purified extract from *M. aurantiaca* strain TMC-15

DPPH radical scavenging assay

The flavones (eupatilin and hydroxyauranetin) effect on DPPH radical scavenging is shown in Table 1, increased with concentration of the purified extract. 80% scavenging activity was offered by flavones when used at concentration of 50 µg µl⁻¹. Ascorbic acid was run in parallel as positive control, showed 97% scavenging activity in pure form.

Lipid peroxidation and protein carbonylation assays

The role of flavones (eupatilin and hydroxyauranetin) against oxidative damage to lipid and protein was also studied. The oxidative effect on protein (BSA) and lipid (rat liver homogenate) was inhibited 73% and 51%, respectively. The standard ascorbic acid (50 µg µl⁻¹) showed 79% inhibition (Table 1).

Iron chelation assay

Oxidative damage of cellular component due to free available iron in the body is extensively linked to degenerative diseases. These free Fe^{+2} can generate superoxides through Fenton pathway. The electron donating potential of such compounds are also very affective in metal ion chelation as shown in Table 1 (87%) when compared with standard EDTA.

DNA damage protection assay

Several metabolic processes generate toxic superoxides and other active OH species. These freely available O and OH ions attack on DNA resulting oxidative damages. DNA damages have extensively studied for its relevance to pathogenesis of multiple diseases. Here, we investigated the DNA preventive effect of eupatilin and hydroxyauranetin using plasmid pUC18 with H_2O_2 , FeSO_4 and sodium nitroprusside. The gel electrophoresis results indicated that oxidative damage to plasmid pUC18 was inhibited by flavones as shown in figure 5.

Computational studies

Molecular docking analysis

The docking protocol was validated first by docking the co-crystallized ligands at the site reported in the crystal structure. The results revealed similar compounds conformation both in docking and crystal structures thus validating the accuracy of docking protocol. For 5-hydroxyauranetin, the top docked conformation has binding affinity of $-3.2 \text{ kcal mol}^{-1}$ to 1N8Q and -7.0 kcal/mol for 1OG5. The eupatilin binding potential for 1N8Q and 1OG5 is $-4.1 \text{ kcal mol}^{-1}$ and $-7.5 \text{ kcal mol}^{-1}$, respectively. The interactions of compound to receptor molecules at the docked positions are provided in Figure 6. Eupatilin complexes with their target enzymes illustrates strong hydrogen bonding and intramolecular ligand Van der Waals interactions as shown in figure 7.

Molecular dynamics simulation

Both compounds in complex with the enzymes (1N8Q and 1OG5) were subjected to dynamics understanding. In general, all the four complexes depicted very stable behavior as demonstrated by the C α -RMSD. This concludes strong intermolecular binding and stable conformation of the compound with the receptors. The mean C α -RMSD computed for the complexes is: 1N8Q-5-Hydroxyauranetin (1.7 Å), 1N8Q-Eupaltin (2.2 Å), 1OG5-5-Hydroxyauranetin (1.5 Å), and 1N8Q-Eupaltin (1.8 Å). Additionally, C α -RMSF of all complexes were estimated to examine residual flexibility of the enzyme residues in the compound presence.

In order to get insights of conformation stability, the RMSD of compounds was plotted versus simulation time. As shown in figure 8, the compounds original docked conformation is quite stable.

MMPB/GBSA analysis

The docking affinity of the compounds for the enzymes was tested further using popular binding energy methods of MMGBSA and MMPBSA. All the four complexes demonstrated rigorous binding energies as

tabulated in Table 3. To the net energy of complexes, significant binding energy contribution was noticed from gas phase in both MMGBSA and MMPBSA whereas the solvation energy contribution is likely to be less important in complex formation. In the solvation phase, non-polar energy somewhat plays a role in binding contrast to the polar solvation contribution.

Table 3
Binding energies of the docked complexes.

Method	Energy Component	1N8Q-5-Hydro-xyauranetin	1N8Q-Eupaltin	10G5-5-Hydro-xyauranetin	10G5-Eupaltin
MMGBSA	VDWAALS	-52.82	-57.51	-42.35	-50.2933
	EEL	-269.83	-32.26	-30.66	-36.5203
	EGB	280.48	47.40	50.64	48.5143
	ESURF	-7.27	-6.44	-5.57	-5.5441
	DELTA G gas	-322.66	-89.78	-73.02	-86.8136
	DELTA G solv	273.21	40.95	45.07	42.9702
	DELTA TOTAL	-49.45	-48.83	-27.94	-43.8434
MMPBSA	VDWAALS	-52.82	-57.51	-42.35	-50.2933
	EEL	-269.83	-32.26	-30.66	-36.5203
	EPB	267.61	55.64	46.66	61.2405
	ENPOLAR	-3.76	-3.55	-3.97	-3.7890
	EDISPER	0.0000	0.0000	0.00	0.0000
	DELTA G gas	-322.6620	-89.7878	-73.02	-86.8136
	DELTA G solv	263.8484	52.0852	42.69	57.4516
	DELTA TOTAL	-58.8136	-37.7026	-30.3279	-29.3621

Discussion

Thal desert of Pakistan has been characterized by high solar radiation, salinity and wide temperature fluctuation between day and night. There is no available literature to explore these extreme environments in search of new strains able to produce valuable compounds. Most of the isolates characterized in our study were able to produce colored colonies on solid agar medium. The chromogenic factor of these microbes might be helpful in their protection against UVB induced oxidative damage. Previously a number of studies have been carried out to isolate radio-resistant bacteria from desert soil, the radiation resistant property is acquired as a result of evolution that protect cells from desiccation (Rainey et al.,

2005). The ability of these UVR microbes to survive in several extreme conditions, is suggested to be as a result of modulating gene expression in extreme conditions (Li et al., 2014) thereby producing colored compounds, shield the bacterium in stress. There is also a strong correlation between mitomycin C, H₂O₂ resistance and secondary metabolite production. The ability of radioresistant microbes to survive in extreme conditions is suggested to be a result of three combined mechanisms: prevention, tolerance and repair. The results also suggested that TMC-15 has a strong catalase and superoxide dismutase antioxidant system thereby protecting the cell from oxidative damages and showed prolong resistance to all three variables.

The phylum actinobacteria is ubiquitous and considered one of the most valuable eco-friendly prokaryotes in field of microbial biotechnology. Recently, methanolic extract from genus *Micromonospora* isolated from extreme hyper-arid Atacama Desert soil evaluated for biotechnological potential (Carro et al., 2019). There are several putative genes of *Micromonospora* that cope stress responses (Trujillo et al., 2014; Brettin et al., 2015) such as heat shock, cold shock, desiccation, and oxidative stress. *M. aurantiaca* has genes associated with SOS repair and also superoxide dismutase genes. In recent years, better understanding of ROS-induced oxidative damages and searching for new bioactive molecules and novel strategies to protect the cellular damages are the two major goals of medical research. Our results revealed that intracellular eupatilin and hydroxyauranetin, confer protection against oxidative stress conditions. There are no previous reports on eupatilin and 5-hydroxyauranetin in radio-resistant bacteria. The results from *in-vitro* biological activities suggested that the intracellular extract from strain TMC-15 scavenges free radicals, comparable to properties reported for radio resistant *Deinococcus* strains (Slade et al., 2011; Sajjad et al., 2017). The strong scavenging potential of these compounds may also be due to conjugated double bond and resonance structure which can easily transfer or donate electron to form final stable product. The presence of keto, hydroxy groups and active fractions like quercetin and gallic acid analogs contributing to such antioxidant activities (Yang et al., 2011).

Flavones have potential as chemotherapeutic drug for different pathological conditions related to free radicals. The antioxidant results offered by these flavones against free radicals generated by FeSO₄ in protein oxidation inhibition assay are due to the capability of such compounds to donate electrons thereby neutralizing the free radicals. In our study the flavones extracted from this bacterium was more efficient in preventing the protein and lipid oxidative damages as studied in BSA and mice liver homogenate (Prazdnova et al., 2014). The kosmotrophic nature of the studied flavones also aids to decrease the solubility of different cellular membranes and strengthen the intermolecular hydrogen bonding to maintain the integrity of membranes and prevent leakage of cellular contents (Krinsky and Johnson 2005). Due to conjugation double bond system, resonance structure and presence of keto groups, such compounds result in the formation of relatively stable end products which suggests that it can be effectively used in drugs and for other therapeutic applications.

The biological significance of iron and predominantly its pathological concerns are due to its involvement in the oxidation-reduction process known as the HaberWeiss reaction where it generates different super oxides that have deleterious effects on the cells, resulting in mitochondrial damage, DNA oxidation and

peroxidation of membrane lipids. Moreover, the free iron can react with unsaturated lipids resulting production of alkoxyl and peroxy radicals. The consequences of iron-overload are apparent in diseases such as thalassemia, Friedreich's ataxia and other pathological conditions that lead to cancer (Heli et al., 2011; Kolnagou *et al.*, 20014). Through a series of reactions these flavones through oxidation-reduction mechanism form stable end products thereby prevent the cells from oxidative damage by blocking the Fenton reactions and by quenching super oxides. Iron chelation therapy signifies a novel strategy of treatment for these disorders, preventing oxidative effects by removing catalytically active iron. The gel electrophoresis results indicated that oxidative damage to plasmid pUC18 was inhibited by flavones. The possible mechanism might be due to the inhibition of 7,8-oxo-2-deoxyguanosine formation generated in stress (Sajjad et al., 2017). This study also showed that many other DNA repair enzymes, cellular proteins, enzymatic antioxidants are protected by these flavones. The protective ability of the studied flavones contributes to prolong the cell survival in extreme conditions and significantly reduces the DNA damage risk (Singh and Gabani 2011). Eupatilin and 5-hydroxyauranetin have significant industrial and pharmaceutical importance, and now there is a potential for producing them in bioreactors.

The antioxidant potential of purified compounds was additionally investigated computationally to decipher their binding affinity to selected receptors. Both compounds bound exactly to the same site of the enzymes and interact with common set of residues. The 5-hydroxyauranetin to 1N8Q formed two strong hydrogen bonds with Ile857 and His518 additional to strong network of hydrophobic contacts. This compound prefers to interact hydrophobically with the enzyme residues. Eupatilin complexes with their target enzymes illustrates strong hydrogen bonding and intramolecular ligand van der Waals interactions as shown in figure 7. It was observed that in the presence of compound, enzyme residues in all four complexes are significantly stable with exception to 1N8Q-Eupaltin where Asp20-Phe40 region showed higher fluctuations. Upon inspection, fluctuation in this region is the outcome of flexible loop covering residue of Pro1-Asn12 and may be an approach to hold the compound strongly at the docked site. The average C α -RMSF of the complexes is: 1N8Q-5-Hydroxyauranetin (0.8 Å), 1N8Q-Eupaltin (1.2 Å), 1OG5-5-Hydroxyauranetin (0.8 Å), and 1N8Q-Eupaltin (0.9 Å). The 1N8Q-Eupaltin as discussed earlier is showing some variation in the binding mode that might be driven by the N-terminal loop to accomplish proper intermolecular binding and enhance complex stability. For each compound, average RMSD with enzyme is: 1N8Q-5-Hydroxyauranetin (0.5 Å), 1N8Q-Eupaltin (0.7 Å), 1OG5-5-Hydroxyauranetin (0.5 Å), and 1N8Q-Eupaltin (0.4 Å).c

Finally, it is concluded that *Micromonospora aurantiaca* strain TMC-15 isolated from extreme environment showed high rate of tolerance to UV, H₂O₂ and mitomycin. The flavones from strain TMC-15 exhibited both antioxidant as well as oxidative damage prevention potential against cellular macromolecules. The docking analysis also revealed significant binding affinity of these flavones to their target proteins through N-terminal loop. Both eupatilin and 5-hydroxyauranetin can be used as potential mitigator and radioprotectants in sunscreen to overcome the radiation mediated oxidative damages. Further studies are needed to determine biosynthetic pathway for enhanced production and intensified bioactivities through metabolic engineering.

Declarations

Funding

This work was funded by Higher Education Commission of Pakistan.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

The current study does not include any human or animal subject; therefore, the ethical committee approval is not required.

Author Contributions

WS, MN, TA and AAS: Preparation of the overall research plan as well as protocols for various experiments

WS, MN, AR: Performed experimental work in lab as per the pre-designed research plan

GD, FH, SK and MB: Facilitated in interpretation of various bioassay and phylogenetic analysis in the current research project

SWA and SA: Contributed in docking and simulation analysis

MF: Analysis of samples through LC-MS and interpretation of LC-MS data.

WS, MN, AR, AAS: Write up of the manuscript

AAS, FH, SK, MB: Proof reading of the overall manuscript for English comprehension and typing mistakes

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Figures

Figure 1

Survival of UV-resistant strain TMC-15 to different oxidative stresses. (A) UV-B radiation resistance potential of strain TMC-15 in comparison to UV-sensitive *E. coli*; (B) Resistance to different concentrations of H₂O₂; (C) Resistance to different concentrations of mitomycin-C

Figure 2

Neighbor-joining phylogenetic tree based on 16S rRNA gene sequencing, showing genetic relationship of bacterial isolate TMC-15(MN721337) to other closely related sequences obtained from the NCBI. The 16S rRNA gene sequence of *Actinomycetales bacterium* was used as an outgrowth. Accession numbers of the sequences are shown in parentheses after the strain name. The numbers at nodes are percentage bootstrap values

Figure 3

FTIR Analysis of purified extract isolated from strain TMC-15

Figure 4

LC-MS chromatogram/negative MS spectrum of purified the metabolic extract. The compound was identified by signals at m/z of 343.9 and 386.9 $[M+H]^-$

Figure 5

DNA damage prevention assay. Lane PC represents positive control containing pUC18. Lane NC displayed negative control without any antioxidant. Sample lane contains pUC18 treated with intracellular extract from strain TMC-15. The gel photo has no letter codes (PC, NC)

Figure 6

Binding mode of the compounds at docked position of 1N8Q (top) and 1OG5 (bottom). The control (protocatechuic acid) in case of 1N8Q (colored as plum cartoon) is shown in tan, whereas as yellow (s-warfarin) in 1OG5 (colored as tan cartoon). Compounds 5-hydroxyauranetin and Eupatilin are presented by sky blue and plum, respectively in both receptors

Figure 7

Chemical interactions of the compounds to the proteins at the docked sites. Coloring of the interactions can be interpreted as light green (van der Waals), red (unfavorable bump), dark green (conventional hydrogen bond), aquamarine (carbon hydrogen bond), purple (pi-sigma), pink (alkyl, pi-alkyl), orange (pi-anion), and fuchsia (pi-pi T-shaped)

Figure 8

MD simulation analysis. Enzymes RMSD in the presence of compounds (A), Enzymes RMSF in the presence of compounds, and compounds RMSD at the docked site of the enzymes (C)

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