Drug target identification and virtual screening in pursuit of phytochemical intervention of *Mycobacterium chelonae*

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Abstract

Mycobacterium chelonae is a rapidly growing mycobacterium present in the environment. It is associated with skin and soft tissue infections including abscess, cellulitis and osteomyelitis. Other infections by this bacterium are post-operative/transplant-associated, catheter, prostheses and even concomitant to haemodialytic procedures. In this study, we employ a subtractive genomics approach to predict the potential therapeutic candidates, intended for experimental research against this bacterium. A computational workflow was devised and executed to procure core proteome targets essential to the pathogen but with no similarity to the human host. Initially, essential *Mycobacterium chelonae* proteins were predicted through homology searching of core proteome content from 19 different bacteria. Druggable proteins were then identified and N-acetylglucosamine-1-phosphate uridyltransferase (GlmU) was chosen as a case study from identified therapeutic targets, based on its important bifunctional role. Structure modeling was followed by virtual screening of phytochemical library (N > 2200), from 500 medicinal plants, against it. A biflavonoid daphnodorin G from *Daphne odora* was screened as having best potential for binding GlmU. Phytotherapy helps curb the menace of antibiotic resistance so treatment of *Mycobacterium chelonae* infection through this method is recommended.

Key words

Mycobacterium chelonae, drug design, phytotherapy, GlmU, virtual screening.

1. Introduction

Mycobacteria species are categorized into two major groups, tubercular and non-tubercular mycobacteria. Nontuberculous mycobacteria are further divided into two groups, rapidly growing and slow growing mycobacteria, depending upon duration of their reproduction in suitable medium (Tortoli, 2014). *Mycobacterium chelonae* is a member of rapidly growing group, which takes less than a week for its reproduction in/on medium. It is the most commonly isolated organism among all rapidly growing mycobacteria. It is mostly found in water sources and on medical instruments such as bronchoscopes (Gonzalez-Santiago and Drage, 2015), and has been isolated from environmental, animal and human sources. *Mycobacterium chelonae* infections in human hosts have increased over time, with reports of both haematogenous and localized occurrences in the recent past (Hay, 2009).

Outbreaks due to *Mycobacterium chelonae* contaminated water and compromised injections are a rising problem. Infections have been linked to cosmetic and surgical procedures, such as trauma, surgery, injection (botulinum toxin, biologics, dermal fillers), liposuction, breast augmentation, under skin flaps, laser resurfacing, skin biopsy, tattoos, acupuncture, body piercing, pedicures, mesotherapy and contaminated foot bath (Kennedy *et al*., 2012; Gonzalez-Santiago and Drage, 2015). *Mycobacterium chelonae* may also colonize skin wounds, as result of which patients form abscess, skin nodules and sinus tracts (Patnaik *et al.,* 2013).

Recent era has observed a trend for search of drug targets in pathogens using computational methods, with a focus on genomic and proteomic data (Shanmugham and Pan, 2013). Comparative/differential and subtractive genomics along with proteomics has been used by many researchers for the identification of drug targets in various pathogenic bacteria like *Pseudomonas aeruginosa* (Sakharkar *et al*., 2004), *Helicobacter pylori* (Dutta *et al.,* 2006), *Campylobacter fetus* (Moolhuijzen *et al*., 2009), *Brugia malayi* (Kumar *et al.,* 2007), *Leptospira interrogans* (Amineni *et al.,* 2010), *Listeria monocytogenes* (Sarangi *et al.,* 2015), *Mycobacterium leprae* (Shanmugam and Natarajan, 2013) etc. Determination of potential drug targets has been made possible by the availability of whole genome and their inferred protein complement sequences in public domain databases (Sarangi *et al*., 2015).

Parenteral antibiotics against *Mycobacterium chelonae* include tobramycin, amikacin, imipenem, and tigecycline, but it has demonstrated resistance to antibiotics and disinfectants (Brown-Elliott

et al., 2012; Jaén-Luchoro *et al.,* 2016). This property assists *Mycobacterium chelonae* in colonizing water systems and allows its access to humans (Jaén-Luchoro *et al.,* 2016). Till now, no specific guidelines for the treatment of *Mycobacterium chelonae* have been defined in the literature (Gonzalez-Santiago and Drage, 2015). This clearly illustrates the need to search out drug targets in the *Mycobacterium chelonae* for design of better therapies against infection by this bacterium, especially using naturally existing metabolites from microbes and plants. In the current study, subtractive proteomics was applied to identify essential druggable proteins in *Mycobacterium chelonae*. Docking of the selected protein GlmU with phytochemicals was carried out for identification of a candidate which might bind it and stop its normal cellular function, leading to bacterial lysis/death.

2. Material and methods

2.1. Prediction of *Mycobacterium chelonae* essential proteome

Complete proteome sequence of *Mycobacterium chelonae* CCUG 47445 with accession no: **NZ_CP007220** was downloaded from the NCBI database. For the prediction of essential proteins, Geptop (Wei *et al.,* 2015) was installed on computer and a search was carried out to align the *Mycobacterium chelonae* protein sequences against the essential or core protein sequences from defined set of 19 bacteria with an essentiality score cut-off value range of 0.15 (Wei *et al.,* 2015). Results were saved and analyzed further.

2.2. Prediction of non-homologous host proteins

In order to find the bacterial proteins which do not have similarity with human host, the set of essential protein sequences of *Mycobacterium chelonae* was subjected to BLASTP against the human proteome database (Uniprot release 2014). The standalone BLAST software (Altschul *et al.*, 1990) was used for this purpose. For identification of non-homologous proteins, an expectation (E-value) cut-off of 10^{-2}, gap penalty of 11 and gap extension penalty of 1 was set as the standard. E-value cut-off (10^{-2}), based on reported research protocols (Perumal *et al*., 2007; Sarangi *et al.,* 2015) was considered.

2.3. Identification of putative drug targets

There are several molecular and structural properties which have been explored by researchers for selecting suitable therapeutic targets in pathogenic microorganisms. These properties include determination of molecular weight, sub-cellular localization, 3D structure and druggability analysis (Hassan *et al*., 2015; Silverio-Machado *et al.,* 2014; Uddin *et al.,* 2015). These properties

were evaluated for selection of the therapeutic targets in *Mycobacterium chelonae*. Molecular weight was calculated by using computational tools and drug target-associated literature available in the Swiss-Prot database (Boeckmann *et al.,* 2003). Subcellular localization of therapeutic targets was predicted using PSORTb (Nancy *et al*., 2010). It uses feature support vector machine-based method and suffix tree algorithm for downstream analysis. Predictions are grouped through a Bayesian scheme into one final (consensus) result. Druggable targets were identified with BLAST hits through unified protocol from the DrugBank. Parameters were: gap cost: -1 in case of extension or opening, mismatch penalty: -3, E-value: $1* 10^{-5}$, match: 1, filter algorithm: DUST and SEG (Azam and Shamim, 2014). The targets were subjected to KEGG blast for identification of associated pathways.

2.4.Virtual screening of ligand against selected target

Keeping in view the results of sub-cellular localization, molecular weight determination and druggability analysis, an essential protein (GlmU) was chosen for further downstream processing. Swiss Model was used for the prediction of 3D structure of the selected target protein (Biasini et al., 2014). This tool constructs structure model by recognizing structural templates from the PDB using multiple threading alignment approaches (Wang *et al*., 2016). The top structure used for structure prediction was that of GlmU from *Mycobacterium tuberculosis* (PDB ID: 3D8V). The structure was validated and analyzed for quality using SAVES (https://services.mbi.ucla.edu/SAVES/), consisting of ERRAT, VERIFY3D and Ramachandran plot analysis.

A phytochemical library consisting of 2266 phytochemical compounds was then docked with GlmU (Ashfaq *et al*., 2013; Mumtaz et al., 2016). Docking was carried out using Molecular Operating Environment (MOE) with the parameters: placement: triangle matcher, rescoring 1: London dG, refinement: forcefield, rescoring 2: affinity dG. MOE provides fast and accurate docking results based on dedicated algorithms and accurate scoring functions (Halim *et al.*, 2015). Structural preparation program embedded in MOE added the missing hydrogen atoms, corrected the charges and assigned near precise hybridization state to each residue (Junaid *et al.,* 2016).

3. Results and Discussion

3.1.Essential proteome prediction

Initially, total proteome of *Mycobacterium chelonae* was subjected to core or essential proteins prediction. Geptop identified these proteins by screening against 19 bacteria (Fig. 1), based upon

orthology and phylogeny features. In the process of essential proteome mining through homology-based methods, a query protein is considered as essential if it is also present in another bacterium and experimentally identified as essential for survival. There are various methods for the prediction of essential proteins, for example single-gene knockout, transposon mutagenesis and RNA interference but all these methods are time consuming and laborious. A good alternative is high-efficiency computational methods designed specifically for this type of work (Cheng *et al.,* 2013; Wei *et al.,* 2013). Predicted essential proteins were 305 in number (Supplementary File 1), linked with significant metabolic pathways in the pathogen life cycle and necessary for its survival. In order to disrupt the function and existence of pathogen it is most important to attack those bacterial proteins which regulate important functions (e.g. nutrient uptake) in the host environment (Butt *et al*., 2012). Latest antimicrobial drugs are designed on the principle of the inhibition of the pathogen's metabolic pathway (Lemaitre and Girardin, 2013; Uddin *et al.,* 2015). Therefore, such protein sequences may be considered as possible therapeutic targets.

3.2.Identification of non-host proteins

Non-host proteins refer to those bacterial proteins which do not have homology with human proteins. If the homologous proteins are targeted, they could badly affect the metabolism of host due to similarity with host proteins. Therefore, non-host proteins could be preferred better drug targets, as side effects and cross-reactivity caused by the use of antibiotics could be evaded for harming host (Azam and Shamim, 2014; Sarangi *et al*., 2015). Among the core proteome of *Mycobacterium chelonae*, 117 proteins (Supplementary File 2) indicated 'no hit' against the human proteome according to the set criteria. These proteins were then used for subsequent analysis.

3.3.Drug Target mining and analysis

BLASTp was performed to identify significant drug target from newly selected essential proteins. Only 17 proteins had significant hits against druggable proteins present in the DrugBank (Table 1).

Molecular weight determination and druggability analysis could improve the screening process for therapeutic targets, as observed previously for numerous pathogenic bacteria and fungi (Abadio *et al.,* 2011). The molecular weight for each potential drug target was calculated (Table 2) and based upon previous studies, it is suggested that smaller proteins are readily soluble and easier to purify (Duffield *et al.,* 2010).

Sub-cellular localization is a critical factor as it helps in accessing the target gene. Cellular functions are compartment specific, so if the location of unknown protein is predicted then its function could also be known which help in selection of proteins for further study. Membrane proteins are reported as more useful target and more than 60% of the currently known drug targets are membrane proteins (Arinaminpathy *et al*., 2009; Tsirigos *et al.,* 2015). Cytoplasm is the site of proteins synthesis and most of these proteins remain there to carry out their specific functions after synthesis. However, some proteins need to be transported to different cellular compartments for their specific function (Strzyz, 2016). The subcellular localization of non-host proteins of *Mycobacterium chelonae* was predicted and majority were demarcated as cytoplasmic (Table 1).

3.4.GlmU analysis and phytochemical screening

After the characterization of all druggable proteins of *Mycobacterium chelonae,* GlmU was selected for further analysis (out of 305 essential and 117 non-homologous proteins). It is a bifunctional enzyme, exhibiting both acetyltransferase and uridyltransferase activities (Moraes *et al.*, 2015; Sharma *et al.,* 2016).

GlmU has been analyzed for various bacterial species (Patin *et al*., 2015; Rani *et al.,* 2015), including *Escherichia coli* (Brown *et al.,* 1999), *Streptococcus pneumonia* (Kostrewa *et al*., 2001), *Haemophilus influenzae* (Mochalkin *et al.,* 2008) and *Mycobacterium tuberculosis* (Zhang *et al.,* 2009). Predicted structure of GlmU estimated RMSD of 0.5 Å. Structure was found to be a homotrimer. Ramachandran plot showed 97.2% residues in favored regions (90.4% in core, 9.4% in allowed, 0.3% in generously allowed) and no residue in disallowed region. According to VERIFY3D program, at least 80% of the amino acids should have value >= 0.2 in the 3D/1D profile and 96.55% of predicted GlmU residues had an averaged 3D-1D score >= 0.2, thus passing the quality check. An ERRAT score of 90.66 was obtained.

Each monomer of GlmU consists of two domains: N and C-terminal domains. N-terminal domain has α/β like fold, similar to dinucleotide-binding Rossmann fold topology. C-terminal domain exhibits a regular left-handed β-helix conformation and a long α-helical arm connecting both domains (Sharma *et al*., 2016). N-terminal domain is essential for uridyltransferase activity as it catalyses the transfer of uridine monophosphate from uridine-triphosphate to N-acetylglucosamine-1-phosphate (GlcNAc1P). C-terminal domain has acetyltransferase activity as it catalyzes the transfer of an acetyl group from acetyl-CoA coenzyme to GlcN1P, in order to produce GlcNAc1P (Moraes *et al.,* 2015).

GlmU plays fundamental role in the formation of bacterial cell wall by carrying out catalysis of uridine-diphospho-N-acetylglucosamine, an important precursor in bacterial peptidoglycan cell wall (Sharma *et al*., 2016). Activity of acetyltransferase of eukaryotic cells differ from the activity of GlmU in the way that, eukaryotic acetyltransferase occurs on GlcN6P and not on GlcN1P. These properties make GlmU suitable as the drug target (Moraes *et al.,* 2015) and it has been used for drug targeting in bacteria such as *Haemophilus influenza* (Mochalkin *et al.,* 2007) apart from designing inhibitors against it for Mycobacterial species (Li *et al.,* 2011; Tran *et al.,* 2013; Rani and Khan, 2015; Mehra *et al.,* 2016). It is also known that proteins that are involved in more than one pathway of pathogen, in addition to that they are non-host proteins, could be more effective drug targets (Sarangi *et al*., 2015). Inactivation of bifunctional GlmU enzyme leads to loss of mycobacterial viability (Zhang *et al.,* 2008; Rani and Khan, 2015), therefore GlmU was used for docking against phytochemicals.

The possible interaction between protein and the ligand is understood computationally through molecular docking. Docking results of GlmU with compounds from 500 medicinal plants (a phtyochemical library of 2266 compounds (Mumtaz *et al*., 2016), revealed that daphnodorin G was the top scoring compound showing affinity for GlmU (Fig. 3; Table 2). It has molecular weight of 558.495 g/mol and is a metabolite of the plant *Daphne odora* (Taniguchi and Baba, 1996).

Receptor centric docking approach was employed for screening of prospective phytochemical library of compounds from more than 500 medicinal plants against GlmU of *Mycobacterium chelonae.* We focused on phytochemical screening against *Mycobacterium chelonae* GlmU because plant derived/natural compounds could be used as antibacterial therapeutics for treatment of bacterial infections (Aparna *et al*., 2014). A comparative analysis of structural shape and chemical complementarity to GlmU was ranked, based on S value in MOE and the one with least score i.e. Daphnodorin G was obtained as best inhibitor. Analogues of Daphnodorin have been reported previously to show antibacterial and nematicidal activities (Zhuo et al., 2015). Huang et al. (2010) reported Daphnodorin analogs as inhibitor of respiratory syncytial virus while Hu et al. (2000) reported its analog as moderately active against HIV-1. Further Lab testing is proposed to know about minimum inhibitory concentration value and other parameters for Daphnodorin G against *Mycobacterium chelonae.*

4. Conclusion

In this study, we proposed GlmU as one of the important therapeutic drug targets for *Mycobacterium chelonae* as it is bifunctional, essential protein for pathogen and has no homology with human proteome. We have provided putative model for phytotherapy against *Mycobacterium chelonae* through virtual screening-based identification of potent metabolite from a database of more than 2000 compounds. This study could be taken as an initiative for screening and quick designing of phytotherapy against microbes using a computational *modus operandi*. It is expected that our study will also facilitate selection and screening of other *Mycobacterium chelonae* therapeutic target proteins against phytochemical and other relevant compounds for Lab testing and successful entry into drug design pipeline.

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Declaration of interest

The authors declare that they have no conflict of interest.

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Figure/ Table legends

Fig. 1. Workflow describing subtractive proteomics with respective number of obtained sequences. *In silico* parameter analysis included hydrophobicity, sub-cellular location prediction, molecular weight etc.

Fig. 2. (A) Docked GlmU with daphnodorin G (3D conformation) and **(B)** 2D conformation of docked complex showing residue interactions.

Table 1. Predicted druggable proteome of *Mycobacterium chelonae.* GRAVY is average hydrophobicity of the protein measured by Kyte-Doolittle algorithm. Hydrophobicity value below 0 are indicates globularity (means protein is hydrophilic), while score value of above 0 indicates protein to be membranous (hydrophobic).

Table 2. Top 5 compounds obtained with best inhibition score against GlmU of *Mycobacterium chelonae.*

Supplementary File 1. List of bacterial species used for essential proteome prediction of *Mycobacterium chelonae.*

Supplementary File 2. Essential proteome of *Mycobacterium chelonae.*

Supplementary File 3. Non-homologous core proteome of *Mycobacterium chelonae.*

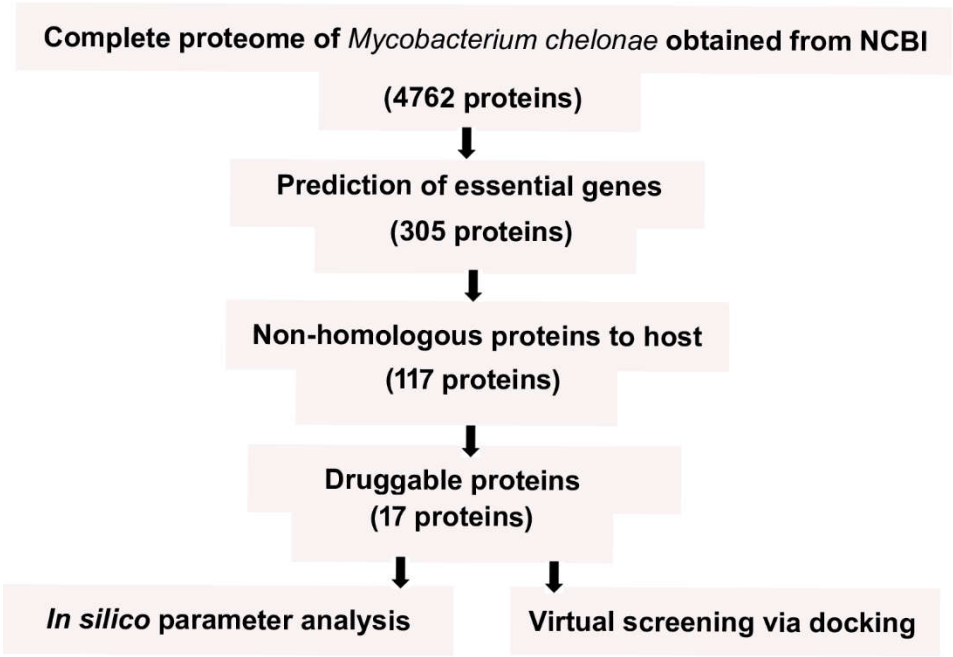
Complete proteome of *Mycobacterium chelonae* obtained from NCBI
(4762 proteins)
Prediction of essential genes
(305 proteins)
Non-homologous proteins to host
(117 proteins)
Druggable proteins
(17 proteins)
In silico parameter analysis
Virtual screening via docking

Fig. 1.

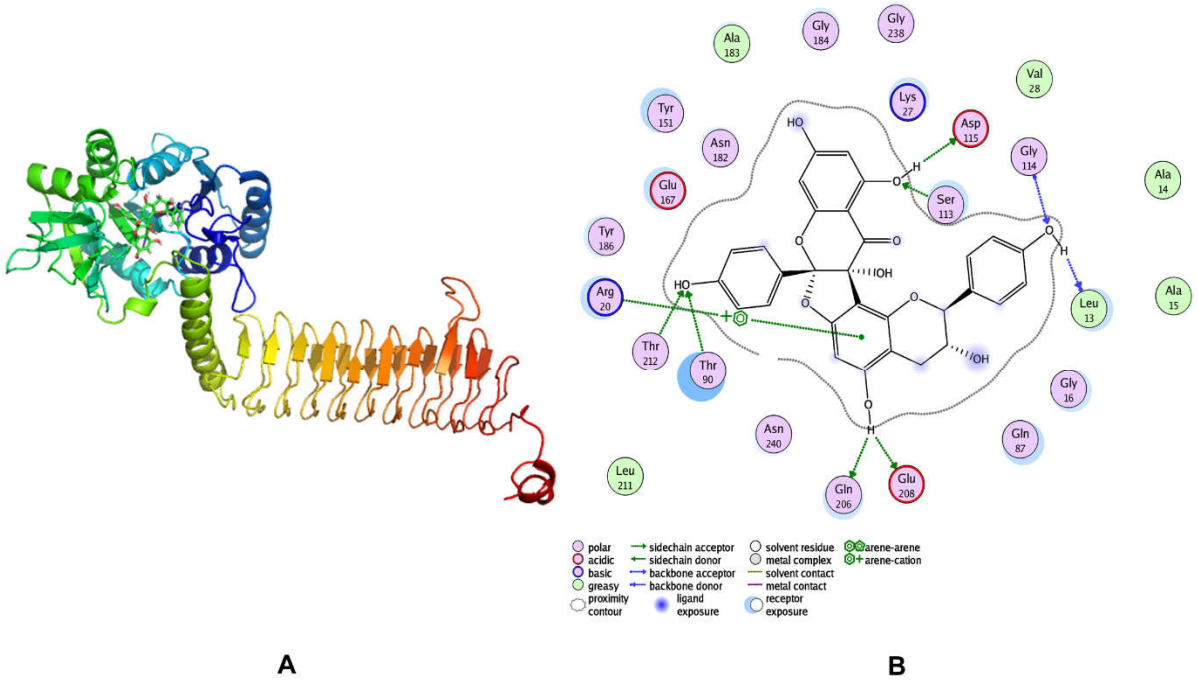
Ala 183
Gly 184
Gly 238
Val 28
Lys 27
Asp 115
Tyr 151
Asn 182
Gly 114
Ala 14
Glu 167
Ser 113
Tyr 186
Arg 20
Leu 13
Ala 15
Thr 212
Thr 90
Gly 16
Asn 240
Gln 87
Leu 211
Gln 206
Glu 208
polar
acidic
basic
greasy
proximity contour
sidechain acceptor
sidechain donor
backbone acceptor
backbone donor
ligand exposure
solvent residue
metal complex
solvent contact
metal contact
receptor exposure
arene-arene
arene-cation
A
B

Fig. 2.

Table 1. Predicted druggable proteome of *Mycobacterium chelonae.* GRAVY is average hydrophobicity of the protein measured by Kyte-Doolittle algorithm. Hydrophobicity value below 0 are indicates globularity (means protein is hydrophilic), while score value of above 0 indicates protein to be membranous (hydrophobic).

Serial No.	Accession No.	Protein name	No. of Matches	Mol. Wt.	Length (amino acids)	GRAVY	Subcellular localization	KEGG Pathway	Experimental evidence for druggability in bacteria
1	**WP_030093515**	6,7-dimethyl-8-ribityllumazine synthase/riboflavin synthase	1	16775.9	162	0.173	Unknown	Riboflavin metabolism, biosynthesis of secondary metabolites	Cushman *et al*., 2002; Cushman *et al*., 2005; Kaiser *et al*., 2007; Zhao *et al*., 2009
2	**WP_030095371**	acyl carrier protein	1	10805.0	99	-0.207	Cytoplasmic	Fatty acid biosynthesis	Madluli *et al.,* 1998; Moche *et al.,* 1999; Heath *et al*., 2000; Wang *et al*., 2006; Luckner *et al*., 2010
3	**WP_030097637**	replicative DNA helicase	1	50372.3	458	-0.323	Cytoplasmic membrane	DNA replication	McKay *et al.,* 2006; Aiello *et al.,* 2009
4	**WP_046251970**	DNA topoisomerase IV subunit B	3	74147.8	675	-0.371	Cytoplasmic	DNA repair and recombination	Hoshino *et al*., 1994; Khodursky and Cozzarelli, 1998; Pan and Fisher, 1998; Anderson *et al.*, 1999; Werner et

									al., 2015; Li *et al*., 2016
5	**WP_046252393**	dihydropteroate synthase	2	37365.3	350	-0.165	Cytoplasmic	Folate biosynthesis	Roland *et al.,* 1979; Hammoudeh *et al*., 2014; Zhao *et al*., 2016
6	**WP_046252395**	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase	1	19332.9	178	-0.054	Cytoplasmic	Tetrahydrofolate biosynthesis	Shi *et al*., 2001
7	**WP_046252399**	pantoate - beta-alanine ligase/ pantothenate synthetase	1	33527.3	314	-0.057	Cytoplasmic	Pantothenate biosynthesis	White *et al*., 2007; Ciulli *et al.,* 2008; Velaparhi *et al.,* 2008; Hung *et al*., 2009; Sledz *et al*., 2010; Yang *et al*., 2011
8	**WP_046252764**	3-oxoacyl-ACP synthase/beta-ketoacyl acyl carrier protein synthase	2	35485.1	340	0.070	Cytoplasmic	Lipid synthesis	He and Rynold, 2002; He *et al*., 2004; Nie *et al.,* 2005; Lee *et al*., 2012
9	**WP_046252769**	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase (GlmU)/glucosamine-1-phosphate acetyltransferase	1	50208.3	482	-0.026	Cytoplasmic	Amino sugar and nucleotide sugar metabolism, Biosynthesis of antibiotics	Pereira et al., 2009; Buurman *et al*., 2011; Stokes *et al*., 2012; Min *et al*., 2012; Mehra *et al*., 2015
1	**WP_046253413**	cell division protein FtsI	1	68842.9	642	-0.300	Cytoplasmic membrane	Peptidoglycan biosynthesis, beta-Lactam resistance	Slayden and Belisle, 2009
1	**WP_046253421**	cell division protein FtsZ	1	38795.6	383	0.076	Cytoplasmic	Chromosome partition, cytoskeleton,	Wang *et al*., 2003b; Ito *et al.*, 2006;

								toxin-anti-toxin	Park *et al*., 2014
1	**WP_046253835**	enoyl-[acyl-carrier-protein] reductase (InhA)	3	28724.0	269	0.152	Cytoplasmic membrane	Lipid biosynthesis	Heath *et al*., 2000; He *et al*., 2007; Luckner *et al*., 2010
1	**WP_046254273**	DNA ligase (NAD(+)) LigA	1	74430.9	684	-0.226	Cytoplasmic	DNA replication, Base excision repair, nucleotide excision repair, mismatch repair	Brötz-Oesterhelt *et al*., 2003; Srivastava *et al*., 2005; Mills *et al*., 2011
1	**WP_046254500**	thymidylate kinase	1	23403.1	212	-0.394	Cytoplasmic	Pyrimidine metabolism	Wang *et al*., 2000; Haouz et al., 2003; Fioravanti *et al*., 2005; Kosinska *et al*., 2005
1	**WP_046254518**	biotin--[acetyl-CoA-carboxylase] ligase	1	27934.5	264	-0.084	Cytoplasmic	Biotin metabolism	Freiberg *et al*., 2004; Polyak *et al*., 2012; Soares da Costa *et al*., 2012
1	**WP_046254614**	alanine racemase	1	41861.3	397	-0.008	Cytoplasmic	D-Alanine metabolism, Vancomycin resistance	Badet and Walsh, 1985; Flynn *et al*., 1985; Anthony *et al.,* 2011
1	**WP_046255923**	ribonucleotide-diphosphate reductase subunit beta/ ribonucleoside diphosphate reductase-β	2	36899.2	320	-0.437	Unknown	Purine and pyrimidine metabolism	Moore, 1969; Tholander and Sjöberg, 2012

Table 2.

Serial No.	Compound	S-value	Metabolite forming plant
1	Daphnodorin G	-7.67	*Daphne odora*
2	Durantanin I	-6.90	*Duranta repens*
3	5,7-Dihydroxy-4'-methoxy-6,8-di-C-methylflavanone 7-(4,6-digalloylglucoside) Matteucinol-7-O-[4",6"-di-O-galloyl]-beta-D-glucopyranoside	-6.15	*Miconia myriantha*
4	Chebulinic acid	-5.89	*Terminalia chebula, Caesalpinia coriana, Phyllanthus emblica*
5	Myricitrin	-5.85	*Pistacia weinmannifolia J.Pisson ex.Franch,* *Rhus parviflora,* *Haplopappus bailahuen,* *Warburgia stuhlmannii,* *Cornus kousa,* *Davidsonia pruriens,* *Doliocarpus spraguei,* *Acacia aroma,* *Acacia saligna,* *Caesalpinia pulcherrima,* *Desmanthus illinoensis,* *Flemingia congesta,* *Quercus rubra,* *Patersonia spp.,* *Juglans mandshurica,* *Leea thorelii Gagnep,* *Myrica rubra,* *Lysimachia spp.,* *Eugenia edulis,* *Luma chequen,* *Plinia pinnata,* *Nymphaea caerulea,* *Nymphaea lotus,* *Phyllanthus emblica,* *Abies amabilis,*

			Armeria sp., *Ceratostigma willmottianum,* *Limonium spp.,* *Eskemukerjea megacarpum HARA,* *Chondropetalum spp.,* *Elegia capensis,* *Diploknema butyracea,* *Manilkara zapota cv.Tikal,* *Leptolaena diospyroidea,* *Leptolaena pauciflora,* *Heuchera spp.,* *Lithophragma spp.,* *Metasequoia glyptostroboides,* *Hedychium spp.,* *Peltiphyllum peltatum,* *Sarcolaeana multiflora*