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Docking studies and network analyses reveal capacity of compounds from *Kandelia rheedii* to strengthen cellular immunity by interacting with host proteins during tuberculosis infection

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Abstract:
Kandelia rheedii (locally known as Guria or Rasunia), widely found and used in Indian subcontinent, is a well-known herbal cure to tuberculosis. However, neither the mechanism nor the active components of the plant extract responsible for mediating this action has yet been confirmed. Here in this study, molecular interactions of three compounds (emodin, fusaric acid and skyrin) from the plant extract with the host protein targets (casein kinase (CSNK), estrogen receptor (ERBB), dopamine β-hydroxylase (DBH) and glucagon receptor (Gcgr)) has been found. These protein targets are known to be responsible for strengthening cellular immunity against *Mycobacteria tuberculosis.* The specific interactions of these three compounds with the respective protein targets have been discussed here. The insights from study should further help us designing molecular medicines against tuberculosis.

Keywords: *Kandelia rheedii*, Emodin, Fusaric acid, Skyrin, Casein kinase, Estrogen receptor, Dopamine beta hydroxylase, Glucagon receptor

Background:
Kandelia rhedii, alternatively known as *K. candel,* is a mangrove plant found abundant in Indian subcontinent- especially in Bengal deltaic region. It falls into the family named Rhizophoraceae **[1]**. It is a small evergreen tree and heights up to 6 m and much branched **[2-6]**. Leaves of this plant are imparipinnate; leaflets range between 3 to 5 per leaf. Leaves are firm, 3.8-6.3 cm long, distant, alternate and suborbicular. The plant has small flowers- pale yellow in axillary panicles and shorter than the leaves. Pods are 3.8-10 cm long, lanceolate **[7]**.

Kandelia rheedii (locally known as Guria or Rasunia) is a well-known herbal cure to tuberculosis. Several small molecules, for example Skyrin, Fusaric acid and Emodin, from the plants have been reported up till now. Skyrin, a fungal bisanthroquinone, exhibits functional glucagon antagonism by uncoupling the glucagon receptor from adenylate cyclase activation in rat liver membranes **[8]** Fusaric acid is a picolinic acid derivative **(Supplementary Figure)**. It is typically isolated from various Fusarium species, and has been proposed for a various therapeutic applications. Fusaric acid is an important antibacterial agent and can also be used to kill cancer cells **[9-15].** It thus can be used as a biocontrol agent **[16, 17]**. Emodin is a purgative resin, 6-methyl-1,3,8-trihydroxyanthraquinone. Emodin is being studied as a potential agent that could reduce the impact of type2 diabetes **[18].**

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The Casein kinase protein kinases are serine/threonine-selective enzymes. They function as regulators of signal transduction pathways in most eukaryotic cell types **[19]**. Estrogen receptors are a group of proteins found inside cells. They are receptors that are activated by the hormone estrogen. Once activated by estrogen, the estrogen receptor is able to bind to DNA and regulate the activity of many different genes **[20].** Dopamine β-hydroxylase (DBH) is an enzyme that converts dopamine to norepinephrine. It is expressed in noradrenergic nerve terminals of the central and peripheral nervous systems, as well as in chromaffin cells of the adrenal medulla. Norepinephrine, released from sympathetic neurons, and epinephrine, released from the adrenal medulla, participate in a number of physiological processes including those that facilitate adaptation to stressful conditions. The thymus, spleen, and lymph nodes are richly innervated by the sympathetic nervous system, and catecholamines are thought to modulate the immune response. However, the importance of this modulatory role *in vivo* remains uncertain **[21].** The glucagon receptor is activated by glucagon and is a member of the class B G-protein coupled family of receptors, coupled to G alpha receptor **[22].**

Several drugs are prevalent in treating tuberculosis. Most common drugs used today are- Isoniazid **[23]**, Pyrazinamide **[24-26]**, Ethambutol **[27, 28]** and Rifampin **[29-31]**. Isoniazid, which works as a prodrug, gets activated by bacterial KatG (catalase-peroxidase) enzyme **[32]** and works on bacterial enoyl-acyl carrier protein/InhA. This hampers the mycolic acid, a fatty acid in the *Mycobacterium. spp.* Cell wall, synthesis for the bacteria and thus prevents further propagation of it **[33]**. Pyrazinamide diffuses into *M. tuberculosis* and it is enzymatically converted into active form pyrazinoic acid which accumulates in the bacterial cell **[34]**. Pyrazinoic acid inhibits fatty acid synthase (FAS) and also reported to inhibit translation of the dormant bacteria **[35]**. Ethambutol also works by obstructing the formation of cell wall. It inhibits arabinogalactan synthesis by blocking arabinosyl transferase enzyme and thereby inhibits cell wall synthesis **[28, 36]**. Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase **[37].**

A tuberculosis drug may work specifically against the bacterial proteins or may act to strengthen the host immune system itself. Until recently the drugs targeting the bacterial proteins seemed to be functioning well **[38].** However, with rapidly emerging resistant strains, it appears that the potential of drugs that enhances host immunity by binding host proteins are also of great import. The small molecules found here from natural sources have been shown to bind to host proteins and thereby strengthen host immunity. Natural plant extracts, in many cases, have been reported to be strong agents that boost host immunity **[39, 40]**.

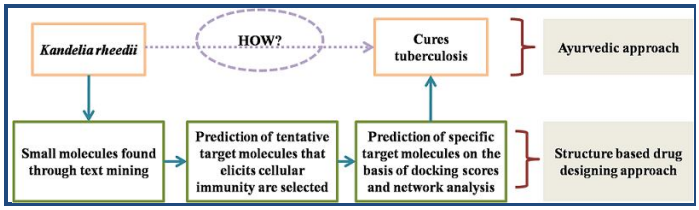

Figure 1: Graphical representation of the methods applied in the study. From ayurvedi studies it has been reported that *Kandelia rheedii* can cure tuberculosis. However, the ayurvedic approach fails to explain the mechanism how it does so. Structure based drug designing approach, as outlined in this study, predicts how in the molecular level this action is carried out.

Methodology:

A graphical representation of the methods applied in the study has been illustrated **(Figure 1).**

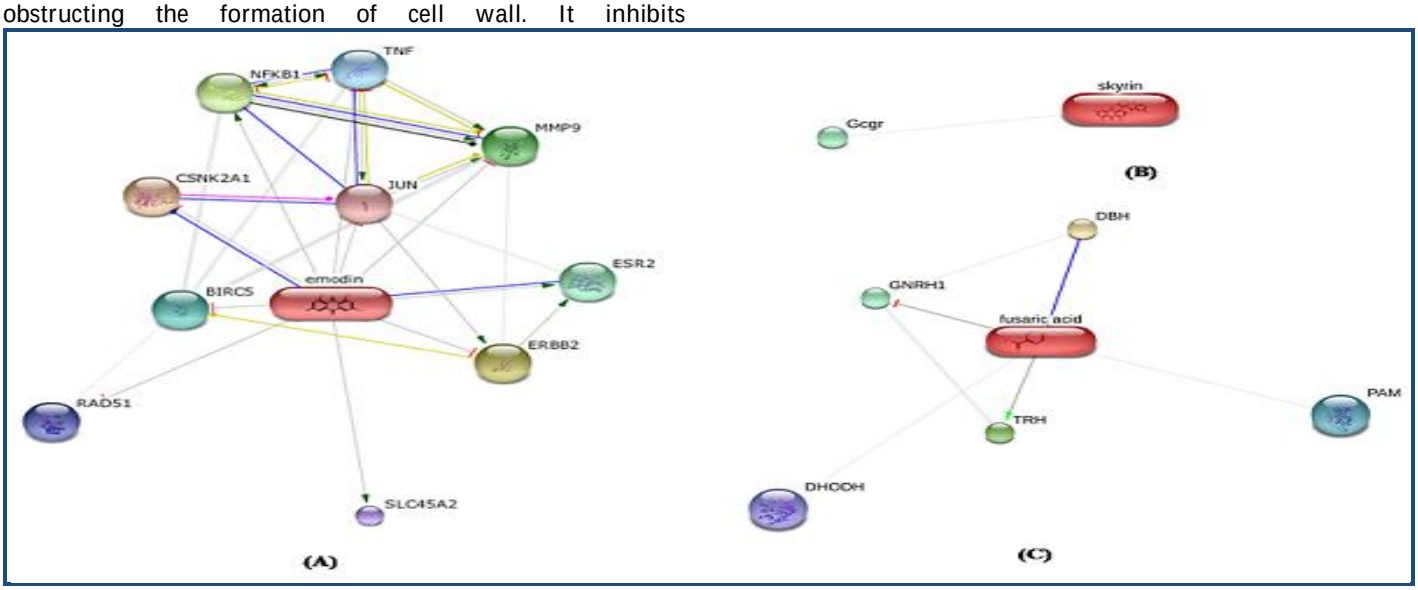

Figure 2: Probable interactors for the ligands (A) Emdin can interacts to different molecules to either induce (arrowhead) or suppress (flat line) them; **(B)** Skyrin can interacts with Glucagon receptor; **(C)** Like emoldin Fusaric acid too can interacts with many different molecules.

Ligand identification

The ultimate goal of the study was to propose active compounds in the plant extract and their molecular mechanism. To find the abundant compounds in the plant several databases and literatures were looked into. An online database titled **Medicinal Plants of Bangladesh** (http://www.mpbd.info/)

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was used to retrieve essential information on the plant. Several encyclopedias were also used for the purpose. Among them some notable ones were C. P. Khare edited **Indian Medicinal plants** and Yifan Yang edited **Chinese Herbal Medicine: Comparisons and characteristics**. Literature search and data-mining also revealed key information on the abundant compounds of the plant.

Target identification

The abundant small molecules were at first searched at **STITCH 3.1** (http://stitch.embl.de/) **[41]** databases for their corresponding interactors. The database returns probable interactor targets on the basis of text mining. Therefore the not much about the binding interaction of the ligand and the target molecules can be predicted from the database results. Still the results were helpful as a guide to carry out docking studies. **ImmPort** (https://immport.niaid.nih.gov/) and **KEGG (Kyoto Encyclopedia of Genes and Genomes)** (http://www.kegg.jp/) **[42-46]** databases were used for characterizing the interacting proteins. ImmPort database returns potential immune related protein targets for the small molecule. Proteins with ability to induce cellular immunity were chosen as the potential candidate for having anti-tuberculosis activity. The **Reactome** database **[47]** retrieved the mechanism how the selected target proteins may mediate their action.

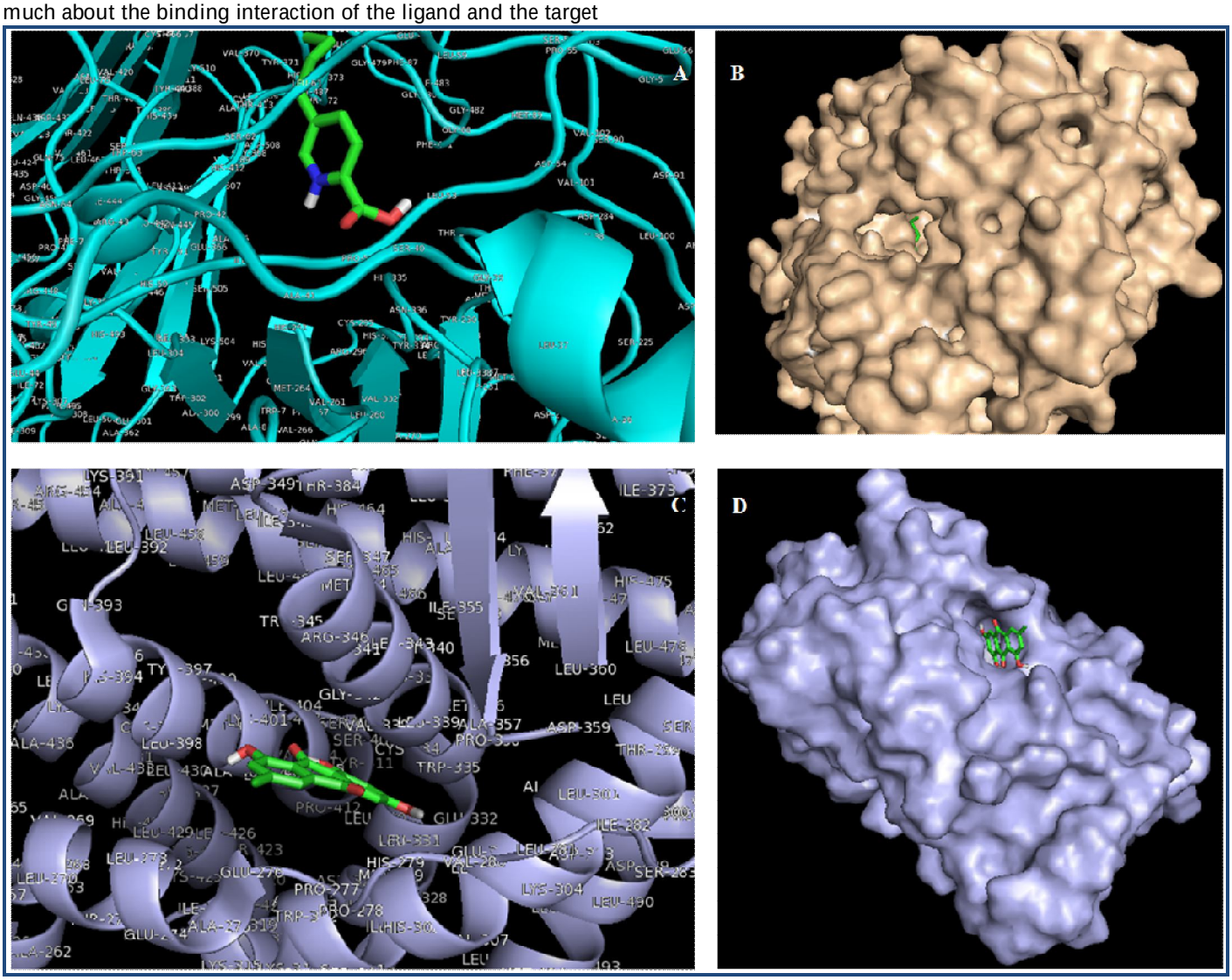

Figure 3: Small molecules binding to the target proteins. (A) Fusaric acid binds to DBH in a grove that is larger in size than the space required for the binding. Cartoon/ribbon representation of the structure is shown; **(B)** Fusaric acid structure is well protruded inside the groove. The target molecule is shown in surface representation and the small molecule in stick; **(C)** Emodin binds to ERBB protein in a groove surrounded with helixs structures. Many of the polar amino acid residues (R346, H279, E276) in those helices regions are responsible for stabilizing the small molecule structure; **(D)** The protein is illustrated as cartoon structure and the pocket is well visualized in this illustration.

Docking

The 3D ligand structure was downloaded from online database **ChemDB** (http://cdb.ics.uci.edu/) and was subsequently docked to protein 3D structure of the macromolecule. Target molecule sequences were collected from NCBI Proteins database. The macromolecule 3D structures were simulated using **I-TASSER** (zhanglab.ccmb.med.umich.edu/I-TASSER/) online server**.** To ensure propriety of the docking results a

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control experiment was run. In the control experiment random small molecules were made to bind to a specific target or random small molecules were made to bind to the specific target molecule. If the binding values for the target and the ligand was higher than that of the random controls the docking scores were deemed to be significant.

Ligand and macromolecule was docked using two programs- **AutoDock Vina** (Download link: http://vina.scripps.edu/download.html) and online tool **PATCHDOCK** (http://bioinfo3d.cs.tau.ac.il/PatchDock/).

Analysis

The ligand bound protein structures after docking were viewed by **RasWin** and **PyMol [48].** The AutoDock results were analyzed using **AutoDock tools -1.5.6rc3** and PyMol. Protein pockets, candidates for ligand binding sites, were found by **DogsiteScorer** and **Pocket Finder** tools.

Results:

From text mining Skyrin, Fusaric acid, Emodin, Norlichexanthone and Secalonic acid were found to be the potential active compounds for the plant **[49-56].** Norlichexanthone and Secalonic neither bound to any *Mycobacterium tuberculosis* proteins nor did it bind to any host proteins. Additionally, the STITCH 3.1 database search predicted tentative target molecules for Skyrin, Fusaric acid and Emodin. The found interactors were all host proteins and no interacting *Mycobacteria spp*. specific target molecules were found. Among the tentative target molecules found, the host proteins that elicit cellular immunity were chosen as potential targets **[57].**

For the ligands, several tentative target molecules in host were found. For Emodin Casein kinase, V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, Matrix metallopeptidase 9, Estrogen receptor, Baculoviral IAP repeat-containing 5, Tumor necrosis factor, RAD51 homolog, solute carrier family 45, Jun oncogene were found to be potential target molecules. For Fusaric acid Dopamine β-hydroxylase, Thyrotropin releasing hormone Fragment, Gonadotropin releasing hormone 1, Peptidyl-glycine alpha-amidating monooxygenase Precursor, Dihydroorotate dehydrogenase, Mitochondrial precursor protein was found to be potential target molecules. For Skyrin only Glucagon receptor Precursor was found to be a potential target molecule. However, only Casein kinase and Estrogen receptor for Emodin, Dopamine β-hydroxylase for Fusaric acid, Glucagon receptor Precursor for Skyrin was known to showed any association with cellular immunity. Cellular immunity plays central role in curing tuberculosis. Therefore, these four tentative targets were shortlisted for further docking study. The summary of the found association for these four proteins are enlisted in **[57-68] (see supplementary material)** and **(Figure 2).** Most of these predicted target molecules generally show good correlation with inflammation process.

The docking studies help to predict specific target molecules from the tentative ones. Emodin binds to casein kinase and the binding affinity was -7.1 kcal/mol- a value much higher than the control values. CSNK binding was further validated by looking for active sites in the groove where the small molecule binds. The groove turned out to fall into the active pocket as predicted by DogSiteScorer. Emodin was also made to bind to ERBB and the binding affinity score for that was -7.4 which is higher than the control values **Table 3 (see supplementary material).** Fusaric acid was made to bind to DBH and the results found were surprising. For Fusaric acid binding the scores were -6.2. The active site for Fusaric acid was also on the most predominant pocket**.** However the area of Fusaric acid binding was much lower than that of the available binding space. The binding affinity of Skyrin to Gcgr gave good scores. The random binding scores were too lower than that of the Skyrin to Gcgr score of -7.7 **(Table 3)**. The overall binding affinities and binding affinities for the control is enlisted in **(Table 3).** All docking results were duplicated using PATCHDOCK online docking tool. The results of PATCHDOCK correlated well with that of AutoDock **(Figure 3).**

The mechanisms of the four target molecules were predicted using Reactome database. The database returned potential interactors and the reactions carried out by the target molecules. Based on the database information a molecular network was generated for all the four predicted targets. Network for Casein kinase revealed that it is responsible for inhibiting ceramide transport from the endoplasmic reticulum to the Golgi apparatus. This results in an upregulation in Wnt pathway. Wnt pathway causes the cellular immunity to improve **Table 2 (see supplementary material)**. On the other hand, Estrogen receptor is directly linked to chemokine signaling pathway regulated by YAP trabscription factor which causes an upregulation of immune response **Table 1 & 2 (see supplementary material)**. It also may act as pro-apoptic agent in infected cells **(Table 2**). Dopamine β-hydroxylase (DBH) causes a general upregulation in the cellular immunity by causing rapid neurostimulation **(Table 1** & **2)**. The molecular networks reveal that Skyrin binds to the glucagon receptor and triggers a G protein linked signaling pathway.The G protein linked pathway then activates an Adenylaye Cyclase (AC) which leads to cAMP production and Protein Kinase A (PKA) production as a consequence. PKA induces a wide array of cellular activities including ion channel opening and transcription factor mediated gene induction. This results in accelerated metabolic activity of the immune cells and thus causes immunity against tuberculosis **(Table 1 & 2)**.

Discussion:

The study selected abundant compounds of the herbal plant by mining a wide array of literatures, encyclopedia as well as databases and consequently predicts target molecules for those selected molecules. *Kandelia rheedii* has long been found to have anti-tuberculosis activity **[69, 70]**. There have been many literatures that reported the active products of the plants. However the molecular mechanism how the plants extract mediates the action is still unclear. This study tries to answer this question using computational analysis.

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*- a bacterial pathogen that can survive and persist in the human host even amidst robust immune response. In tuberculosis the bacteria infects one of the major immune cells- macrophage. Since, this cell is a major inflammation mediator; in TB often having robust humoral immune response does not improve the

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situation for the patient. The most effective way of fighting this disease has been to improve the cellular immunity as a whole **[71]**.

Cellular immunity is the major immune pathway that is activated to fight out tuberculosis infection. Major effectors of cellular immunity are the T cells. T cells can be of two types- one is of CD4+ and MHC-II recognizing T_H cells and the other one is CD8+ and MHC-I recognizing Tc cell. It has been shown that both the cells play their parts in protecting host cells against the bacterial infection **[72]**.

T_H or T-helper cells are the lineage of T cells that secret several cytokines and thus ameliorate inflammatory and antibody mediated immune response against both infected cells and the bacteria itself. On the other hand, T_C or cytotoxic T cells are responsible for killing infected macrophage cells **[73, 74]**.

A prophylactic often blocks the specific enzymes of the causative pathogens **[38]**. Drugs that are being used currently against tuberculosis are also designed against bacterial proteins. However, as an alternative mode of action the drug can also act on the host proteins and bolster the host immunity **[39].** The small molecules found here from natural sources have been shown to bind to host proteins and thereby strengthen host immunity. This paves the way for an alternative way of designing prophylactic drugs against *Mycobacteria tuberculosis* **[40].**

Molecular network study has become a major way of understanding the molecular mechanism of drug action nowadays **[75-77].** Network based drug discovery has become a recurrent theme for natural compounds too; characterization of the drug regulated genes and targets have been assisted greatly by computational techniques **[78-80].** Rational drug designing from natural sources have made drug costs and required resources to go down significantly over the years **[81-83].** Hence this study holds great implications for drug designing from natural products **[84-86]**.

The results suggest that the Emodin, one of the most copious small molecules in the plant extract, binds to two target molecule- Casein kinase and Estrogen receptor. Casein kinase is responsible for up-regulating Wnt pathway and it consequently improves cellular immunity. In addition it also binds Estrogen receptor and hence accelerates chemokine signaling pathway which results in a rapid upregulation of inflammatory mediators **(Table 1)**. Another small molecule- Fusaric acid, on the other hand, binds Dopamine β-hydroxylase (DBH) and causes a non-specific intensification of cellular immunity **(Table 1)**. In a similar note, Skyrin binds to the Glucagon receptor which triggers a cascade of reaction keeping G protein linked receptor at the center of it **(Table 1)**. All three proteins hence play both specific and non specific roles in inducing the cellular immunity. Their association to cellular immunity is enlisted in **(Table 1).**

According to the docking studies carried out, Emodin is predicted to be a potential ligand of CSNK. Skyrin also seem to be a potent ligand of Glucagon receptor. However, not much of a specific correlation was was found between the target molecule of Skyrin and tuberculosis. From these studies only Emodin could have been predicted as the mediator molecule without much doubt.

Docking study for herbal drugs has been applied successfully in last few decades **[87-89].** Since AutoDock has been used widely for drug designing **[90, 91**], the predictions made in this study seems to be computationally valid. However, only further *in vitro* studies can absolutely confirm the predictions.

A significant revelation was made in case of Fusaric acid binding in DBH. The depth to area ratio for the binding pocket was (77.20 / 9149.91 =) 0.00843 **(Data not shared)**. The area of the groove was quite large with respect to the height. This may open up a new perspective. Often ligands that are buried well inside the protein have some sort of channeling process **[92-94].** Emulating the channeling computationally could have improved the docking score. However, in docking studies, despite the flexibilities of induced fit, it is hardly possible to emulate this effect as the experiment is done with one constant structure. Only further *in vitro* studies can confirm whether there is any channeling effect there in that protein or not.

The control small molecules were drawn at random from PubChem database. The control target molecule structures too were taken at random from PDB database. The random control ensured the propriety of the prediction and ruled out high score values generated due to discrepancies.

There have not been many studies to connect among ayurvedic, allopathic and molecular medicines. Ayurvedic drugs put more focuses on strengthening host immunity as a whole whereas allopathic focuses on treating the pathogenic agent [38-40]. Both the concepts are important in designing molecular medicines. Designing drugs, against both host and pathogenic macromolecules, on the basis of structure and function holds momentous importance in molecular medicines **[95].** However, with a growing popularity of natural medicine this integration has never been more likely in the coming days **[95].** The study tries to establish the molecular basis of the traditional herbal extract and thus employs an integrated approach. The study should further be useful for designing synthetic drugs against *Mycobacteria tuberculosis.* It is to be noted that often promising computational analysis fail to get reproduced *in vitro* and hence the study must have to be further validated *in vitro* before a conclusive remark.

Conclusion:

The extracts from the plant *Kandelia rheediihas* has been traditionally used in the treatment of tuberculosis. However, the molecular mechanism of such action was not known for compounds isolated from the plant extract. Here, it is shown that Emodin binds to Casein Kinase (CSNK) and Estrogen receptor (ERBB), Fusaric acid binds Dopamine β-hydroxylase (DBH) and Skyrin binds Glucagon receptor (Gcgr) using molecular docking and network analysis. There have not been many studies to bridge among ayurvedic, allopathic and molecular medicines. In this regard, the study attempts to fill this gap. However, the results need to be tried *in vitro* for conclusive proof of concept.

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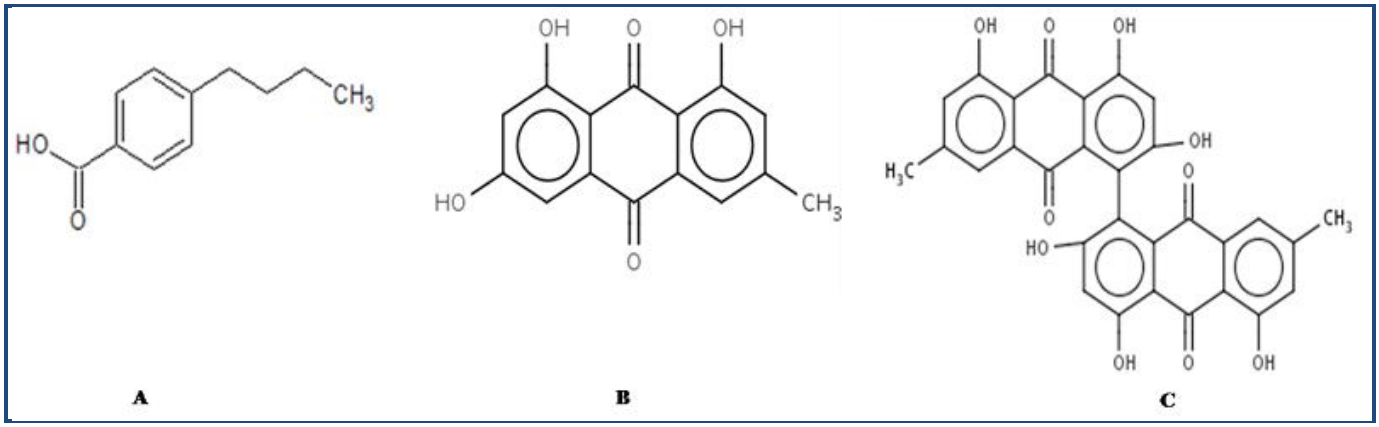
Supplementary material:



Supplementary Figure: Structure of the compounds. The structure of Fusaric acid **(A)**, Emodin **(B)** and Skyrin **(C).** Fusaric acid posseses a butyryl group and a carboxyl group at para position to it added on a benzene ring. Emodin has a quinine structure and Skyrin has a poly cyclic structure.

Table 1: Summary of the target molecule selection on the basis of association to tuberculosis protection

Target molecule	Pathway	Coherence to cellular immunity
Casein kinase (CSNK)	Wnt signaling Hedgehog signaling pathway Gap junction Circadian rhythm in mammal Adherens junction Tight junction	Restore cellular inflammation in microbe induced inflammation [57] Alteration in tight junctions during tuberculosis [58]
Estrogrn receptor (ERBB)	Steroid hormone biosynthesis Chemokine signaling pathway Focal adhesion Leukocyte transendothelial migration Regulation of actin cytoskeleton Bacterial invasion of epithelial cells Sulfur metabolism	TLR mediated inflammatory response [59] Altered sulfur metabolism [60]
Dopamine β-hydroxylase (DBH)	Cellular immunity Neurosignaling	Pro-inflammatory response [61]
Glucagon receptor (Gcgr)	Autoimmune response Neuroactive ligand-receptor interaction	TLR mediated inflammatory response [62]

Table 2: Mechanism of action as predicted for selected target proteins

Target molecule	Action	Consequence
Casein kinase (CSNK)	monophospho-CERT + 2 ATP => multiphospho-CERT + 2 ADP	This reaction has the effect of inhibiting ceramide transport from the endoplasmic reticulum to the Golgi apparatus as multiphospho-CERT is unable to bind ceramides or associate with the Golgi membrane[63].
Estrogrn receptor (ERBB)	Transmembrane receptor for estrogen. TACE mediated proteolytic cleavage turn it into a transcription factor.	The C-tail of ERBB4 possesses several WW-domain binding motifs (three in CYT1 isoform and two in CYT2 isoform), which enable interaction of ERBB4 with WW-domain containing proteins. ERBB, through WW-domain binding motifs, interacts with YAP1 transcription factor, a known proto-oncogene, and may be a co-regulator of YAP1-mediated transcription. [64] Once in the mitochondrion, the BH3 domain of ERBB4, characteristic of BCL2 family members, may enable it to act as a pro-apoptotic factor [65]
Dopamine β-	Catecholamine	Norepinephrine, released from sympathetic

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hydroxylase (DBH)	biosynthesis where dopamine is oxidised to noradrenaline.	neurons, and epinephrine, released from the adrenal medulla,participate in a number of physiological processes includingthose that facilitate adaptation to stressful conditions. [66-68]
Glucagon receptor (Gcgr)	Transmembrane receptor for glucagon.	In response to low blood glucose, pancreatic alpha-cells release glucagon. The binding of glucagon to its receptor results in increased cAMP synthesis, and Protein Kinase A (PKA) activation.PKA mediated phosphorylation:PKA phosphorylates key enzymes, e.g., 6-Phosphofructo-2-kinase Fructose-2,6-bisphosphatase (PF2K-Pase) at serine 36, and regulatory proteins, e.g., Carbohydrate Response Element Binding Protein (ChREBP) at serine 196 and threonine 666. Dephosphorylated ChREBP activates the transcription of genes involved in glucose metabolism such as pyruvate kinase, and lipogenic genes such as acetyl-CoA carboxylase, fatty acid synthetase, acyl CoA synthase and glycerol phosphate acyl transferase

Table 3: Binding affinities for small molecule binding to their respective target molecules

Binding	Highest binding affinity (kcal/mol)	Average control value for random target	Average control value for random small molecule
Emodin bound to CSNK	-7.1	-6.6	-6.4
Emodin bound to ERBB	-7.4	-6.6	-7.1
Fusaric acid bound to DBH	-6.2	-5.2	-6.2
Skyrin bound to Gcgr	-7.7	-6.8	-6.5

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