

Isolation, Characterization & In-Vitro Anti – Tubercular Activity Of *Epiphyllum Oxypetalum* Leaf Extract

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ABSTRACT

Epiphyllum oxypetalum, which belongs to the family Cactaceae, is characterized by the fact that it is a nocturnal ornamental leaf whose culture has been known to be powered by white leaves. The plant is native to the rainforests of Central America and it has also captured attention because of its important medicinal qualities. In phytochemical examination of its leaf extracts, bioactive compounds like alkaloids, flavonoids, terpenoids and saponins have been found to be responsible in contributing to diverse pharmacological activities associated with the herbal compound. The plant has shown good prospects in antimicrobial, anti-inflammatory, antioxidant and anticancer. One such subject of interest has been in its effectiveness against *Mycobacterium tuberculosis* (M. tuberculosis), the pathogen causing tuberculosis (TB), a health burden all over the globe because of the increasing drug resistance of the strains. The in vitro experiments have revealed that extracts of *Epiphyllum oxypetalum*, especially ethanolic extracts demonstrate a strong anti-TB effect, which is likely to be associated with the presence of the compounds capable of interfering with bacteria cell walls and the processes of metabolism. With the growing threat of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, plant-based treatment options, like *Epiphyllum oxypetalum* hold a promising prospect of coming up with new kinds of treatment strategies. Future studies of active compounds isolation, in vivo testing and clinical trials are essential to determine the entire treatment potential of the said plant against drug-resistant TB.

Keywords: Tuberculosis (TB), Drug resistance , multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, High performance thin layer chromatography (HPTLC) , Microplate Alamar Blue Assay (MABA) , Anthranilate phosphoribosyltransferase (PDB ID: 3R6C).

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease, which is induced by *Mycobacterium tuberculosis* and which usually attacks the lungs, but may also attack other body organs like kidneys and brain. TB is contracted by inhaling airborne sputum of sick patients and the symptoms include persistent cough, fever, night sweats and weight loss. TB has been a leading killer of people across the world despite the development of antibiotics, and drug resistant strains such as MDR-TB and XDR-TB continue to make treatment challenging. Low- and middle-income countries suffer the most burden of the disease with a huge number of cases in such countries as India and South Africa, where overcrowded living conditions

and insufficient healthcare structures add to the issue. The onset of TB-HIV co-infection has also contributed to the increased morbidity and mortality due to TB thus complicating the fight against TB.

Epiphyllum oxypetalum or the Queen of the Night is a Central American cactus that has immense cultural and medicinal importance. It is a known plant used traditionally across several cultures to treat respiratory diseases, wounds, and inflammation and is known to have beautiful fragrant flowers that bloom overnight. Its bioactive properties such as alkaloids, saponins, flavonoids, and terpenoids are part of its therapeutic activity with anti-bacterial, anti-oxidant properties and anti-inflammatory properties. It has been noted that ethanol extracts have anti-TB effects

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and may be used as a natural remedy to drug-resistant strains, which has recently been identified as a promising potential anti-TB agent because of its ability to inhibit *Mycobacterium tuberculosis*.

Although *Epiphyllum oxypetalum* is also a promising agent in anti-TB, its commercialization as a therapeutic agent is hampered by factors such as the necessity to establish its viability in vivo and its molecular mechanisms. Although the plant is easily accessible and has multifunctional medicinal values, the plant provides a wide range of compounds with variation in yield and requires a consistent quality measure makes it difficult to scale up to the clinical level. Nevertheless, this can be overcome through further studies on the isolation of its active compounds and the ability to test their efficacy, which might generate newer, alternative therapies to treat TB, especially as more cases worldwide grow resistant to drugs.

MATERIALS AND METHODS

1. Sample Collection and Extraction:

The *Epiphyllum oxypetalum* leaf was collected and authenticated by a botanist making sure that the leaf was correctly identified before the extraction process was undertaken. This sample was then dried in shade and kept aside to dry further. It was performed in terms of extracting bioactive compounds based on the maceration technique with three most-used solvents, namely, ethanol, chloroform and aqueous solutions. The coarse leaf powder was provided in a 25g quantity and each solvent was added to this separately in the quantity of 100ml. These blends were set on an orbital shaker with 24 hours' rotation rate at 32 °C temperature, with room temperature. The solvent types were designed in such a way that they represented different polarity indices that could see that many different phytochemicals could be extracted in the leaf. The mixtures were filtered with Whatman filter paper after the extraction period in order to get rid of solid residues. It came up with three independent extracts, that is, ethanol, chloroform and aqueous with different contents of the bioactive compounds depending on the type of the solvent employed. These extracts were kept in store to be further analyzed which might involve the test of their pharmacologic characteristics, i.e. antimicrobials,

antioxidants or anti-inflammatory. The fact that various solvents are used will make sure that various types of phytochemicals are extracted and that they possess different potential uses in therapy.

2. Phytochemical Analysis of *Epiphyllum oxypetalum* Extract:

- The phytochemical analysis of *Epiphyllum oxypetalum* leaf extract revealed the presence of several bioactive compounds through various tests:
- **Alkaloids:** The presence of alkaloids was confirmed using Wagner's test, which showed the formation of a reddish-brown precipitate when the extract was mixed with Wagner's reagent, indicating alkaloids are present.
- **Carbohydrates:** The reducing sugars in the extract were detected using Fehling's test, where a red precipitate formed upon boiling the extract with Fehling solution, indicating the presence of reducing sugars.
- **Glycosides:** Borntrager's test was used to detect glycosides, with the chloroform layer turning pink after adding ammonia solution, confirming their presence in the extract.
- **Saponins:** The foam test was conducted by shaking the extract with distilled water, and the formation of foam indicated the presence of saponins.
- **Proteins:** Biuret's test was performed by adding copper sulfate, ethanol, and potassium hydroxide to the extract, and the appearance of a pink color in the ethanolic layer confirmed the presence of proteins.
- **Phenolic Compounds:** Phenolic compounds were detected using the ferric chloride test, where the addition of ferric chloride resulted in a dark green color, indicating their presence.
- **Terpenoids:** The presence of terpenoids was confirmed by adding sulfuric acid to the



chloroform extract, and a reddish-brown layer formed, indicating the presence of these compounds.

- **Steroids:** Steroids were detected by adding acetic acid and concentrated sulfuric acid to the extract, with a blue-green color change confirming their presence.
- **Flavonoids:** Flavonoids were identified by adding ammonia solution to the extract, and the formation of a yellow color indicated their presence.
- **Tannins:** The presence of tannins was confirmed by adding ferric chloride solution to the extract, which resulted in a green color, indicating the presence of tannins.
- These results show that *Epiphyllum oxypetalum* contains a variety of bioactive compounds, which support its potential therapeutic effects, including antimicrobial, anti-inflammatory, and antioxidant activities. Further studies are needed to isolate and fully characterize these compounds for more detailed pharmacological analysis.

3. High-Performance Thin Layer Chromatography:

The high-performance thin layer chromatography (HPTLC) plate was prepared by placing 1 gram of silica gel in 60 ml of distilled water to make an even suspension and then applied on the surface of the plate and allowed to dry at room temperature. The stationary phase was the silica gel and the mobile phase was a mix of solution of benzene, ethyl acetate, and acetic acid (5:1.5:0.25 ratio). A thin band was used to apply the sample with the help of an automatic spotter and the plate was developed in a chamber during a period of about 10 minutes. Following the development, the plate was analyzed with a UV lamp at 256 and 364 nm to determine the compounds according to the mobility and the strengths of the bands. The most promising bands were taken forward to further purification and characterization with a view to studying their pharmacological relevance.

4. Column Chromatography:

Column chromatography was the method used to separate the phytochemicals with silica gel as the stationary phase because it is a polar compound. The first step consisted in preparing a suspension of silica gel (30 g) with hexane and then pouring it in a column with cotton at the bottom in order to avoid the escape of the adsorbent. There was column filling with hexane, so there was no air pockets. After setting up the column, 5 ml of the extract was added and it was eluted by adding hexane and ethyl acetate in different proportions (10:0, 9:1, 8:2, etc.) to the mobile phase to increase its polarity. This change of polarity assisted the separation of the compounds according to their affinity to the stationary and mobile phases. This was repeated until the column was colorless, and therefore fully separated. Fractions collected were further characterized.

5. In vitro Anti-Tubercular Assay by MABA Method

Preliminary screening of extracts was conducted against *Mycobacterium tuberculosis* reference strain H37Rv using the Microplate Alamar Blue Assay (MABA) at concentrations of 250 mg/mL, 500 mg/mL, and 1000 mg/mL. Rifampicin (32 µg/mL) was used as a positive control, and sterility controls were included. The MABA method, which uses resazurin as a redox dye, measures microbial growth by color change from blue (no growth) to pink (growth). Extracts were tested in 96-well microtiter plates, with two-fold dilution series performed across the rows. After incubation, the color change was observed to determine bacterial growth, and the Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration that prevented the color change. The results provided insights into the antimicrobial efficacy of the extracts, indicating their potential as anti-tuberculosis agents.

6. In-Silico Molecular Docking Studies

Anthranylate phosphoribosyltransferase (PDB ID: 3R6C) 3D crystal structure of the tuberculosis protein was obtained at the RCSB PDB database. The protein surface properties and where it might interact with other proteins were analyzed using CASTP and PyMOL. Compounds were filtered using the Lipinski Rule of Five (molecular weight <500 g/mol, log P <5, 0 or fewer hydrogen bond acceptors, 0 or fewer



hydrogen bond donors, and 0 or fewer rotatable bonds) and were further studied. The results of these compounds were then exposed to docking experiments to determine their affinity with the tuberculosis protein. The discovered phytoconstituents of the GC-MS analysis were utilized as ligands, and their respective 3D structures were obtained via the PubChem database. Molecular docking was done using Molegro Virtual Docker (MVD) which optimizes the prediction of ligand-protein interaction based on the Piecewise Linear Potential (PLP) scoring mechanism. The MVD program was used to determine the best binding positions, identification of hydrogen bonds, and determination of the ligand energy with respect to the electrostatic interaction. Detection of the cavities and search algorithms based on the differentials evolution led to the optimization of the docking process. The analyzing of the ligand interactions and binding affinities was made possible by using the Pose Organizer to analyze the returned docking poses. MolDock score of individual ligands was then correlated to a standard drug; the best ligands, which had the highest MolDock scores, were considered as potential lead molecules in the treatment of TB. The outcomes of such docking process give an insight into the relevance of the compounds towards the targeting of tuberculosis proteins.

RESULTS AND DISCUSSION

1. Sample Collection and Extraction:

The preparation of the *Epiphyllum oxypetalum* leaf extract was performed by the method of maceration with solvent liquids of different polarity (ethanol,

chloroform and aqueous solutions). The solvents together with the leaf material were agitated in an orbital shaker of 24 hours at room temperature. The mixture was left to settle after the extraction period after which filtration through Whatman filter paper was done to remove solid particles. Extracts were obtained and the obtained extracts were dried and contained refrigerator. The approach makes sure that a variety of bioactive compounds, depending on their solubility, are taken and tested further.

2. Phytochemical Analysis:

Phytochemical screening of three different solvent extractions of *Epiphyllum oxypetalum* leaf showed a number of bioactive compounds to include alkaloids, saponins, proteins, terpenoid, flavonoid, tannins and steroids. These components possess different pharmaceutical uses and hence, the leaf extract could be considered a source of therapeutic uses. Alkaloids such as anti-cancer, antimalarial, anti-hyperglycemic activities are known to exhibit pharmacological activities. The extract is made up of saponins that have antibacterial and antioxidant characteristics and have been shown to give the leaf its medicinal potential. Tannins are also known to have antibacterial properties and flavonoids are known to have a good antioxidant activity which aids in the prevention of oxidative damage of body cells due to the toxic free radicals. These compounds were indicated by the initial phytochemical screenings and hence the *Epiphyllum oxypetalum* leaf extract may emerge to be useful in the formation of bio-therapeutic agents to cure a diverse range of illnesses (Table 1). Such results underscore the possible medicinal value of the leaf as a pharmaceutical research.

Table 1: Phytochemical analysis of *Epiphyllum oxypetalum*

TEST	CHLOROFORM	ETHANOL	AQUEOUS
Alkaloids	-ve	+ve	-ve
Carbohydrates	-ve	-ve	-ve
Glycosides	-ve	-ve	-ve
Saponins	-ve	-ve	-ve
Protein and amino acids	+ve	-ve	-ve
Phenol	-ve	-ve	-ve
Terpenoids	+ve	+ve	-ve
Steroids	-ve	-ve	+ve
Flavonoids	-ve	-ve	+ve
Tannins	-ve	-ve	+ve



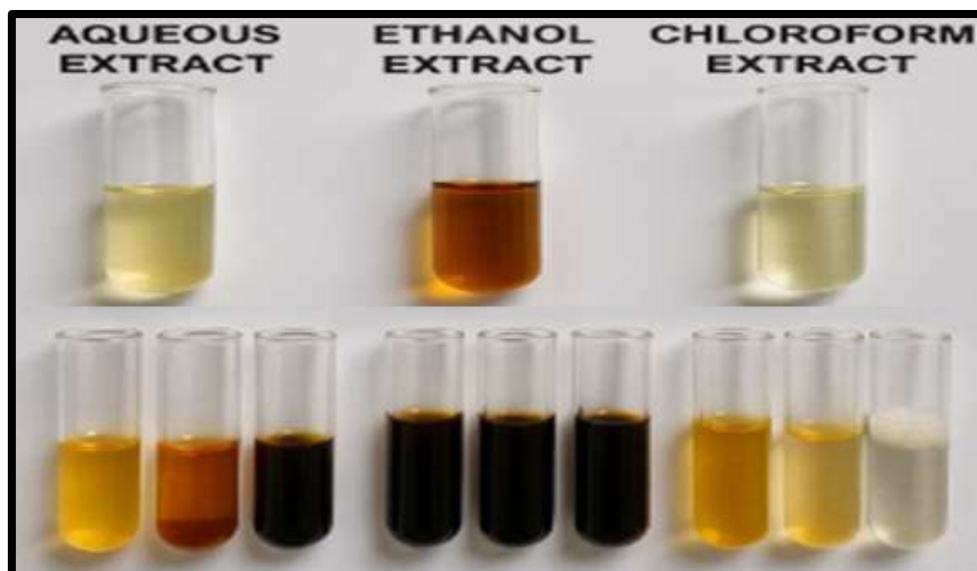


Figure 1: Phytochemical Screening test of *Epiphyllum oxypetalum*

3. HPTLC Analysis:

A High-Performance Thin-Layer Chromatography (HPTLC) was done using the crude *Epiphyllum oxypetalum* leaf extract using various solvents extracts to determine their band formation. Four different bands were observed when the ethanol extract was loaded onto the plate after its development and examination with UV light. The retention factors of the ethanol extract were obtained as 0.35, 0.42, 0.71

and 0.78. The chloroform extract had only one band having Rf value of 0.78 whereas the aqueous extract had two bands, each of the Rf values of 0.71 and 0.78. The highest number of bands based on the number of separated bands and different Rf value were selected which is the ethanol extract, thus, partial purification was done. The specific separation profile of this extract opens up the possibility of further analysis and isolation of bioactive compounds the most (Table 2).

Table 2: HPTLC analysis and number of spots detected

Sample Extract	No. of Spots Detected	Rf Value
Ethanol	4	0.35, 0.42, 0.71, 0.78
Chloroform	1	0.78
Aqueous	2	0.71, 0.78

Compounds Identified:

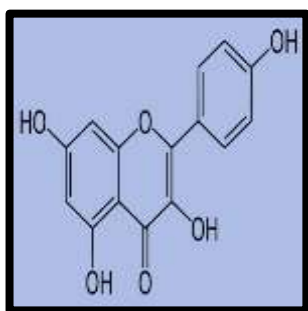


Figure 2: Kaempferol

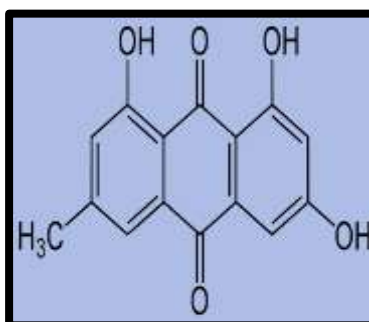


Figure 3: Emodin

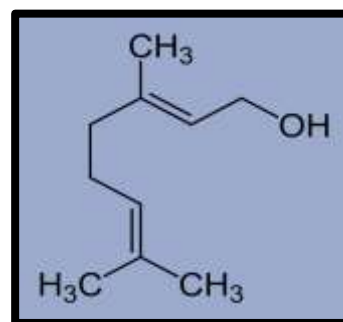


Figure 4: Geraniol

4. Column Chromatography:

Band separation was observed on the TLC plate in three different crude extracts of *Epiphyllum oxypetalum* leaf and the ethanol extract exhibited the improved separation compared to the other two. It was based on this, that the ethanol extract was chosen to be carried forward to a partial purification using column chromatography. 5 mL of ethanol extract containing silica gel was added to the top of the column to which hexane was added as eluting agent.

The solvent ratio of hexane to ethyl acetate (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) was gradually changed to increase polarity of the mobile phase. The fractions that elute were collected and the column could be seen to change color to clear. TLC was used to analyze these fractions and the best separation of bands was observed in the fraction eluted in a ratio of 3:7 hexane ethyl acetate mixture as shown in Figure 5. This proportion was chosen to undergo another analysis and purification.



Figure 5: Column Chromatography of *Epiphyllum oxypetalum*

5. In vitro Anti-Tubercular Assay by MABA Method

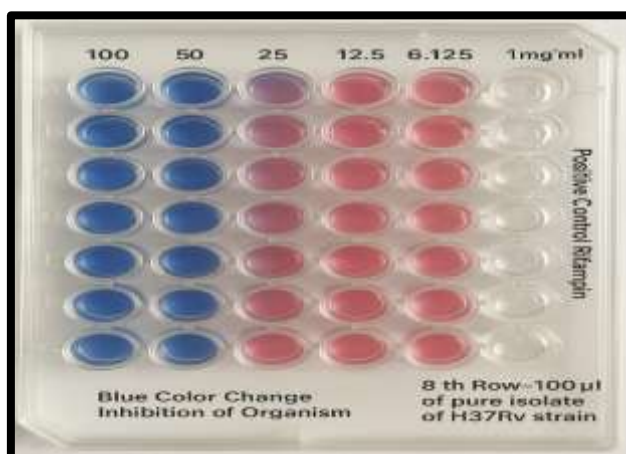


Figure 6: Anti-Tubercular Assay by MABA Method

Columns 1-6: Concentrations of 100, 50, 25, 12.5, 6.125, 1mg/ml. Rows: 7th Row - 40µg/ml of Positive Control Rifampin, 8th Row - 100µl of pure isolate of

H37Rv strain of MTB. Interpretation: Blue Color Change-Inhibition of Organism

6. In-Silico Molecular Docking Studies

MolDock is based on a new heuristic search method that is a hybrid of cavity prediction and differential evolution. The docking scoring function MolDock is a version of piecewise linear potential (PLP) that introduces additional electrostatic and hydrogen bonding terms. The main parameter to explore the results of the docking is MolDock Score.

The assessment of the ligand molecules was done using MolDock score rerank score and interactions of hydrogen bond. Among the ligands, the one that has minimized MolDock score has the highest affinity towards the target. Table 3 shows the table of in silico docking study of the ligands that were selected as against tuberculosis in silico docking.

Table 3: Ranking of Ligands and poses against tuberculosis protein based on MolDock score

Protein: 3R6C

Ligand Id	Ligand Name	MolDock Score	Rerank Score	H Bond
5280863	Kaempferol	-96.4299	-84.8083	-5.58642
3220	Emodin	-91.4938	-86.8303	-16.8034
637566	Geraniol	-77.3445	-64.851	-2.5
3767	Isoniazid (Standard Drug)	-58.8102	-53.8175	-7.21397

2D, 3D and Secondary Interaction between Ligands and 5KIR Protein

A. Kaempferol Vs 3R6C

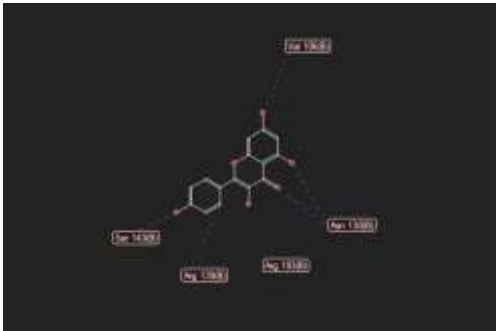


Figure 7: 2D Interaction between Kaempferol with 3R6C

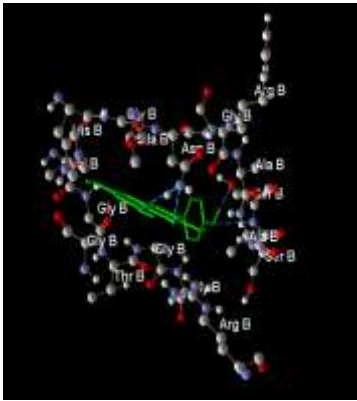


Figure 8: 3D Interaction between Kaempferol with 3R6C

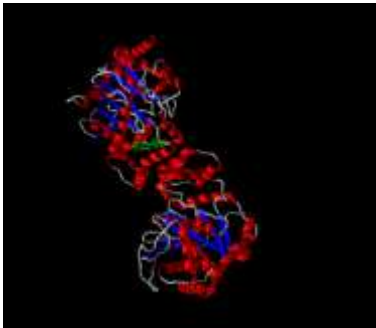


Figure 9: Secondary Interaction between Kaempferol with 3R6C

B. Emodin Vs 3R6C

Figure 10: 2D Interaction between Emodin with 3R6C



Figure 11: 3D Interaction between Emodin with 3R6C



Figure 12: Secondary Interaction between Emodin with 3R6C

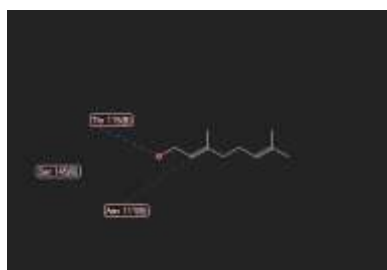
C. Geraniol Vs 3R6C

Figure 13: 2D Interaction between Geraniol with 3R6C

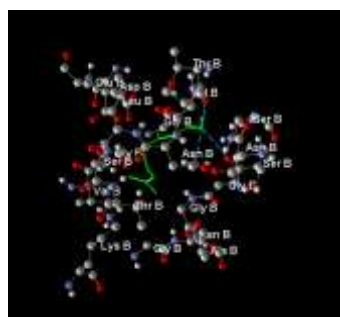


Figure 14: 3D Interaction between Geraniol with 3R6C

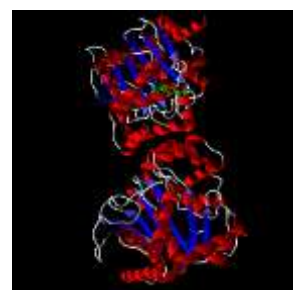


Figure 15: Secondary Interaction between Geraniol with 3R6C

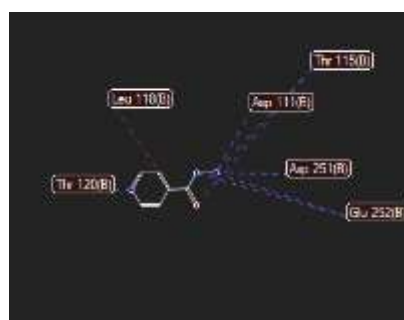
D. Isoniazid Vs 3R6C

Figure 16: 2D Interaction between Isoniazid with 3R6C

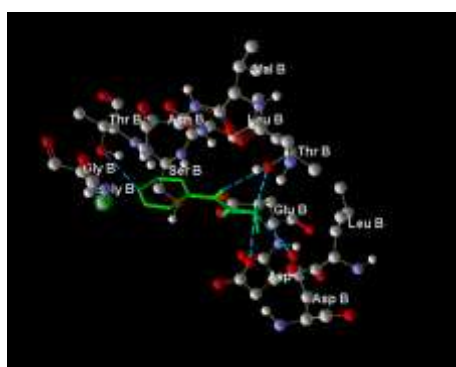


Figure 17: 3D Interaction between Isoniazid with 3R6C

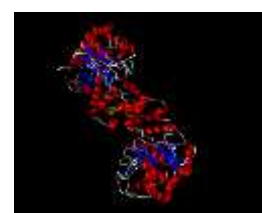


Figure 18: Secondary Interaction between Isoniazid with 3R6C

Table 3 lists four ligands- Kaempferol, Emodin, Geraniol and Isoniazid which were ranked based on MolDock scores with tuberculosis protein 3R6C. As the results indicate, all three phytochemicals of *Epiphyllum oxypetalum* have shown more binding affinity as compared to Isoniazid:

The strongest binding activity was reported with Kaempferol (MolDock score -96.42). Its hydrogen bonding energy was also quite high (-5.58).

- Emodin came as a close second (-91.49), although the HB energy calculated was a strong -16.80 with the potential to have strong hydrogen bonds.
- Geraniol, which was weaker than the other two even scored -77.34 which was better than Isoniazid.
- Isoniazid the reference drug had the most negative binding score (-58.81) and this confirmed that the phytocompounds had the ability to be better candidates.

The figures on interactions (2D, 3D, secondary interaction views) show the binding orientations and modes of each ligand with 3R6C:

- Kaempferol (Figures 7-9) shows several hydrogen bonds and pi pi interactions with active site amino acid residues, indicating such hydrogen bonds might be considered as a stable and specific interaction.
- Emodin (Figures 10-12) exhibits an even greater number of hydrogen bonds, which is in line with its large H-bond score. It has good interactions even in the binding pocket making it a very strong anti-TB agent.

As less interactions are evident with Geraniol it suffices that it has been able to maintain key hydrogen bonds, justifiable to its slightly lower ranking in docking score.

The fact that Isoniazid (Figures 16-18) has a weaker docking score, it has fewer interactions and fewer types of interactions due to fewer hydrogen bonds formed, indicating a shallower binding.

The evaluation of Table 3 and figures 7-18 indicate that Kaempferol and Emodin, which are obtained by *Epiphyllum oxypetalum*, has better binding affinity to the target protein of TB than standard drug which is Isoniazid. Powerful MolDock results and interaction

profiles confirm their value as lead-compounds in the development of an anti-tubercular agent.

DISCUSSION

The Queen of the Night, *Epiphyllum oxypetalum* is a cactus found in tropical rainforest in Central America and Mexico, and is famous because of its spectacular night flowers. Not only has this plant become of interest due to its ornamental value, but also due to its medicinal value. It has found applications in conventional medicine systems such as Ayurveda and Traditional Chinese Medicine on the treatment of disease conditions such as respiratory diseases and skin infections. The plant has several types of bioactive compounds such as alkaloids, flavonoids, terpenoids, saponins and phenolic compounds which make it antimicrobial, anti-inflammatory and antioxidant. These bioactive compounds position *Epiphyllum oxypetalum* as a promising candidate for the development of alternative therapies, particularly against drug-resistant tuberculosis (TB).

The *Epiphyllum oxypetalum* has shown promise as an anti-TB agent in particular in the treatment of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* strains. In vitro testing has indicated that ethanolic extracts of the plant prevent growth of *M. tuberculosis* strains with the lowest concentration (minimum inhibitory concentration or MIC) of 12.5 mg/mL to 25 mg/mL. The most important compounds that include flavonoids, terpenoids, and alkaloids probably cause such effects by destabilizing Gram-negative cell wall, interfering with the ATP production and altering the immune reactions. Also, the plant extracts have not exhibited cytotoxicity, meaning that they are safe to use as therapeutic agent. Nevertheless, the active compounds, which have been identified, should be isolated and characterized, and the efficacy of the plant should be confirmed by in vivo research.

Although the results of the *Epiphyllum oxypetalum* harnessing as a TB clinical therapy are promising, there are still challenges associated with harnessing this plant. It is hindered by the incomplete



characterization of its active compounds, variability in yield of the compounds depending on the conditions of the environment and the necessity to have standard procedures in its extraction. Additionally, in vivo testing is essential to ascertain the safety and effectiveness of the plant in the living organism as a therapeutic agent. However, bioactive compounds in *Epiphyllum oxypetalum* have shown promise in developing novel natural therapies against drug-resistant TB, and further studies may result in the development of new therapeutic agents that would be used to combat this increasing global health menace.

CONCLUSION

In conclusion, *Epiphyllum oxypetalum* has rather a huge potential in serving as the source of bioactive compounds of antimicrobial nature, in particular, of drug-resistant strains of *Mycobacterium tuberculosis*. The presence of multitude of pharmacologically active constituents, such as flavonoids, alkaloids and terpenoids, qualifies the plant as a potentially useful object with regards to additional therapeutic uses. Even though the use in the treatment of TB is founded on the available in vitro studies which are only enough to justify the use of the substance in the environment of anti-TB treatment, there is a necessity to proceed with the in vivo studies which include clinical trials on the determination of the efficacy and safety of the substance under the conditions of treatment of TB. The outcomes have provided a fresh angle of thinking on how the anti-TB vegetable agents can be developed that possibly can be critical in eradicating the global health problem of the multidrug-resistant tuberculosis.

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