VOLUME 9 ISSUE 2 2023

ISSN 2454 – 3055

INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

Forum for Biological and Environmental Sciences

Published by Saran Publications, India

International Journal of Zoological Investigations Vol. 9, No. 2, 699-709 (2023)

International Journal of Zoological Investigations
Contents available at Journals Home Page: www.ijzi.net
Editor-in-Chief: Prof. Ajai Kumar Srivastav
Published by: Saran Publications, Gorakhpur, India

ISSN: 2454-3055

Bioprospecting of Phytoconstituents from Tamarind Pulp and *In Silico* Docking Studies of Flavonol Against Uropathogenic Bacterial Proteins

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Received: 2nd July, 2023; **Accepted:** 22nd September, 2023; **Published online:** 26th September, 2023

https://doi.org/10.33745/ijzi.2023.v09i02.079

Abstract: Urinary tract infection (UTI) is a common bacterial infection among women, where >80% acquire UTI once in their life time and more than 20-50% of them exhibit reoccurrence events where Cystitis (bladder infection) and Pyelonephyritis (Kidney infection) are the predominantly occurring kinds of UTI. The infection is caused by the consequence of bacterial infection in the upper and lower urinary tract. Among the bacterial species *Escherichia coli* contributes 80-85% of the infection followed by *Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis and Staphylococcus saprophyticus.* Due to the recurrence and antibiotic resistance of uropathogenic organisms there is a need for an exploration of an alternative therapy with natural products. The pulp of *Tamarindus indica* is versatile and nutritious which is generally used in Indian cooking as a flavouring agent for its uniqueness in taste (sweet/ sour). It has a wide range of bioactively potential phytocompounds such as phenols, flavonoids, carbohydrates and organic acids. The present study was carried out to analyse the bioactive compounds in tamarind pulp with quantification and characterization (GCMS) of primary and secondary metabolites. The antioxidant studies by DPPH assay has proved the therapeutic potential of the phytocompounds present in tamarind pulp. Further, *in silico* docking analysis was carried out with potential phytocompound (Flavonol) of tamarind pulp as lead compound was docked against the target proteins of Uropathogenic and antibiotic resistant *E.coli* and was compared with the crystalline structure of antibiotic- ciprofloxacin. Stereochemical quality of protein was validated by Ramachandran Plot and quality factor of targeted proteins was analyzed by ERRAT. Further, both the ligand and target were docked using Dockthor with blind docking method. The results revealed that the ligand had a highest binding energy (-6.49 Kcal/mol and -6.62 Kcal/mol) when compared with the standard drug (-7.32 Kcal/mol and -7.27 Kcal/mol) for both the targeted proteins- FmlH gene and CTX-M-15 beta lactamase gene. The results clearly evidence that the role of Flavonol in inhibiting the growth of uropathogenic organisms which may be due to the inhibition of nucleic acid synthesis, DNA gyrase, functioning and permeability of cytoplasmic membrane and the attachment of bacterial pili to the host tissues which in turn inhibit the formation of biofilms through interbacterial interactions by down-regulating the expression of adhesion genes. Addition of appropriate amount of Tamarind pulp in our regular diet having potential flavonoids with its synergistic effect of secondary metabolities would definitely help in the prevention and management of uropathogenic bacteria leading to the occurrence of Urinary tract bacterial infections.

Keywords: DockThor, Flavonol, Phytocompounds, Tamarind pulp, Uropathogenic bacteria

Citation: Thenmozhi P. and Uma Gowrie S.: Bioprospecting of phytoconstituents from tamarind pulp and *in silico* docking studies of flavonol against uropathogenic bacterial proteins. Intern. J. Zool. Invest. 9(2): 699-709, 2023.

https://doi.org/10.33745/ijzi.2023.v09i02.079

Introduction

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting from all age groups and about 250 millions of people around the world every year are being diagnosed by UTI (Sahay, 2020). Due to shortness of urethra, pregnancy and absence of prostatic secretion females are more susceptible than males in which more than 80% of women acquire UTI once in their life time and more than 20-50% of them exhibit reoccurrence events (Lichtenberger and Hooton, 2008; Levison and Kaye, 2013). UTIs are clinically categorised as uncomplicated and complicated UTI. Persons who are healthy and have no structural or neurological urinary tract abnormalities are affected by uncomplicated UTI and are differentiated into Cystitis (bladder infection) and Pyelonephyritis (Kidney infection) (Chee and Maciej, 2016). Risk factors associated with cystitis are diabetes, obesity, vaginal infection, sexual activity and genetic susceptibility. Complicated UTI are associated with factors that comprise the urinary tract or host defence, including urinary obstruction, urinary retention caused by neurological disease, immuno suppression, renal failure, renal transplantation, pregnancy and the presence of foreign bodies such as calculi, indwelling catheters or other drainage devices (Ana *et al*., 2015).

The infection is caused by both gram positive and gram negative bacteria and certain fungi in the upper and lower urinary tract. Among the bacterial species *Escherichia coli* contributes 80-85% of the infection followed by *Klebsiella pneumoniae, Proteus mirabilis, Enterococcus faecalis and Staphylococcus saprophyticus* (Li, 2018). The emergence of resistant strains has posed a big challenge in dealing with UTI and the pattern of resistance also changes based on geographical locations (Seyed Abdol *et al*., 2019; Suhail Ahmed *et al*., 2019). In addition, the continuous use of antibiotics had lead to the emergence of resistance to particular antibiotics (Ana *et al*., 2015). Hence, the empirical treatment and management of UTI have paved a way for the development of new drug with natural products.

Tamarindus indica L. is an evergreen tree with a monotypic genus and species. It is widely distributed in Asia, Africa and America. Every part of Tamarind has been utilised by both rural and urban people for various purposes. Traditionally, the fruits are being used in cooking for its sour and sweet taste but they are also nutritious and have medicinal value (Ei- Siddig *et al*., 2006). According to World Health Organisation, the fruit of Tamarind has all the essential amino acids except tryptophan (Kuru, 2014). The fruit contains important source of mineral elements and the polyphenols present in tamarind pulp have high anti-oxidant and anti-inflammatory property which protect against cardiac problems, cancer and diabetes (Parvez *et al*., 2003).

The sour taste of tamarind pulp is because of tartaric acid- a bioactive compound which is higher in amount having a gentle laxative effect. Apart from medicinal use, it has a wide variety of domestic and industrial purposes in preparing jam, jelly, candy and in paper making (Ei- Siddig *et al*., 2006).

The objectives of the present study were to analyse the phytoconstituents of tamarind pulp both qualitative and quantitatively. To evaluate the Radical scavenging activity by DPPH, characterization of phytocompounds by GCMS and further *in silico* study has been carried out to evaluate the functional role of selected phytocompound against the targeted bacterial proteins.

Materials and Methods

Collection and Preparation of Crude Extracts of Tamarind Pulp:

Ripened tamarind pods were collected from different zones of Tamil Nadu based on soil types (twenty samples codes: CPT-3, CPT-7, CPT-19, CPT-21, CPT-23, CPT-25, CPT-35, CPT-37, CPT-38, CPT-42, CPT-46, CPT-49, CPT- 50, CPT- 51, CPT-55, CPT-56, CPT-64, CPT-65, CPT-66 and CPT-68; CPT = candidate plus tree). The outer shell or husks of the pods were manually removed along with the seeds from the pulp. Further, the ripened pulp has been stored in an air tight container at 4 °C. The fresh tamarind pulp was extracted in the ratio 10: 100 with different solvents selected based on polarity (Petroleum ether- PE, ethyl acetate- EA, acetone- AC, methanol- ME and aqueous- AQ). Later the extracts were used for further analysis.

Phytochemical Screening – Qualitative Analysis:

The different extracts of Tamarind pulp were analysed qualitatively for the presence of primary (carbohydrates, proteins, amino acids and carboxylic acids) and secondary metabolites (alkaloids, terpenoids, phenols, flavonoids, glycosides and tannins) using standard procedures (Raaman, 2006).

Quantitative Analysis of Phenols:

The total phenolic content was measured using the method described by Velioglu *et al.* (1998) with slight modifications. The aqueous and methanol extracts of the tamarind pulp (1 ml) was mixed with 0.5 ml of Folin Ciocalteau reagent. The mixture was incubated at room temperature for 5 min and then 20% sodium carbonate solution was added and incubated for 2 h. The absorbance was read at 760 nm. A standard calibration curve was plotted against different concentrations of gallic acid and expressed as mg of gallic acid equivalents/g of fresh weight.

Quantitative Analysis of Flavonoids:

The total flavonoid content was determined using a calibration curve of quercetin by aluminium chloride method (Pham *et al*., 2007). One ml of the sample (aqueous and methanol extracts) was mixed with 1% aluminium chloride and incubated for 15 min at room temperature. The absorbance was read at 430 nm. The measurement was expressed as mg of quercetin equivalents/g of fresh weight.

Radical Scavenging Activity – DPPH:

Hydrogen donating or radical scavenging activity of the tamarind pulp was determined by the method described by Ana *et al.* (2018) with slight modifications. An aliquot of the extracts of different concentrations (20, 40, 60, 80 and 100 µl) was mixed with 0.1 mM of DPPH. The solution was incubated at dark for 30 min at room temperature and absorbance was read at 517 nm against a blank. Ascorbic acid was used as standard. The per cent inhibition of the sample was calculated as:

$$\% \text{ inhibition} = \frac{\text{Abs. control} - \text{Abs. sample}}{\text{Abs. control}} \times 100$$

Characterization by GC-MS Analysis:

The gas chromatography mass spectrometry was performed at SRM University, using Agilent GC. One µl of sample extract was injected into the instrument with the following parameters, initial oven temperature 60°C for 2 min; followed by an increase of 10°C min^{-1} up to 280 °C. The components obtained from the spectrum were compared with the known components of the database spectrum stored in the GC-MS NIST (2012) library.

In Silico Docking Analysis:

Based on characterization of compounds by GC-MS analysis, the phtyocompound 4h-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6- methyl, a flavonol was selected as the potential ligand for the molecular docking against the proteins FmlH gene and CTX-M-15 which are responsible for causing Urinary tract infection. The 3D structure of the phytocompound (Compound CID: 119838) was retrieved from PubChem and the target proteins FmlH gene (PDB ID: 5LNG) and CTX-M-15 Beta lactamase (PDB ID: 4HBT) from PDB database. The target protein molecules were validated by Ramachandran plot and the non-bonded interactions between the atoms of the target

protein molecules were carried out by ERRAT analysis using PROCHECK server. The ligand and the target protein molecules were docked using DockThor and ciprofloxacin was used as a standard drug.

Statistical Analysis:

The experiments were performed in three replicates. The data were calculated as a mean ± SE, and statistically analyzed using MS Excel by ANOVA, with $p \leq 0.01$.

Results and Discussion

Qualitative and Quantitative Phytochemical Analysis:

Solvent polarity plays a significant role in the extraction process since it enhances the solubility of phytocompounds (Norlia *et al.*, 2014). The qualitative phytochemical analysis of Tamarind pulp using different solvents had revealed the presence of phenols, tannins, flavonoids, glycosides, terpenoids, saponins, carbohydrates, carboxylic acids and Proteins. Amino acids and fixed oil and fats were absent in all the extracts (Table 1). Petroleum ether and Ethyl acetate was found to be the least effective to extract the bioactive compounds from Tamarind pulp which may be attributed by its lowest relative polarity index. Methanol and aqueous solvent had a better extraction of bioactive compounds than the other solvents which may be due to higher polarity index; hence it further enhances its efficacy (Razali *et al*., 2012).

The total phenolic content for aqueous and methanol extracts of 20 samples were carried out, in which the phenol content of aqueous extract ranged from 169.65 $mgg^{-1}$of GAE to 489.06 $mgg^{-1}$of GAE and the methanol extract ranged from 104.77 $mgg^{-1}$of GAE to 413.09 mgg^{-1} of GAE (Table 2). The highest and lowest phenolic content of tamarind pulp were found in CPT-64 and CPT-19, respectively. This revealed that the edaphic factors also play an important role in the content of phytochemicals present in Tamarind pulp (Razali *et al*., 2012).

Biomedical properties of phenolic compounds include antioxidant, anti-inflammatory and antibaterial properties. In addition, it reduces the risk of cancer, cataracts, and hypertension, as well as coronary disease. Antioxidant properties of phenolics are responsible for the preventive effect, along with their interactions with other antioxidants such as vitamin C, carotenoids, folic acid, and fiber (Obulesu and Bhattacharya, 2011).

The results in Table 3 revealed variations in total content of flavonoids ranged from 36.4 mg/g to 350.02 mg/g in aqueous and 12.47 mg/g to 280.22 mg/g in methanol. The total flavonoid content was highest in CPT-64 and lowest in CPT-19. Flavonoids have the C6-C3-C6 structure, which is the primary polyphenol structure. As of now, over 8000 flavonoid molecules have been identified. There are a number of biological activities associated with them, including antioxidant, anti inflammatory, anticancer properties and cardiovascular protection (Bhattacharya, 2011).

Radical Scavenging Activity (*DPPH*):

Excess production of Reactive Oxidative Stress (ROS) are produced during aerobic metabolism in organisms that can lead to oxidative stress, as a result of imbalance between the ROS production and body's ability to counteract with antioxidants. The elimination of these radicals (hydroxyl, peroxide and superoxide) is the primary objective of antioxidant administration (Ziekhrü *et al*., 2023). The formation of radicals in the body leads to aging and the development of diseases like cancer, diabetes and cardiovascular diseases (Pisoschi and Pop, 2015). Consequently, the antioxidant activity of the Tamarind pulp has been analysed by Radical scavenging activity- DPPH.

Antioxidant activities of the aqueous extract of twenty samples are evaluated by free radical scavenging activity along with ascorbic acid (Fig. 1). Among the samples, at 50 mg / ml the highest per cent inhibition (Ic_{50}) was found to be 16.91 in CPT- 21 and lowest per cent inhibition was 42.58 in CPT-65 whereas, the standard ascorbic acid had

Table 1: Qualitative phytochemical analysis of tamarind pulp

S. No.	Phytochemical Constituents	PE	EA	AC	ME	AQ
1	Alkaloids	-	-	+	+	+
2	Terpenoids	-	-	-	+	+
3	Phenols	-	-	+	++	++
4	Flavonoids	-	-	+	++	++
5	Glycosides	+	-	+	+	+
6	Tannins	-	-	-	-	-
7	Carbohydrates	-	+	+	++	++
8	Proteins	-	-	+	+	++
9	Amino acids	-	-	-	-	-
10	Carboxylic acids	-	-	-	+	++
11	Saponins	+	-	-	-	+
12	Fixed oils and fats	-	-	-	-	-

++ Strongly Present, + Present, - Absent

Table 2: Quantitative analysis of phenols

TOTAL PHENOLIC CONTENT (mg of Gallic acid Equivalents/g of fresh weight)		
SAMPLE CODE	**AQUEOUS**	**METHANOL**
CPT- 3	476.87 ± 0.15	397.24 ± 0.15
CPT-7	171.67 ± 0.09	121.93 ± 0.15
CPT-19	169.65 ± 0.15	104.77 ± 0.15
CPT-21	417.76 ± 0.15	376.91 ± 0.15
CPT-23	294.61 ± 0.15	233.93 ± 0.15
CPT-25	211.39 ± 0.15	175.21 ± 0.15
CPT-35	330.39 ± 0.15	299.31 ± 0.15
CPT-37	387.77 ± 0.15	341.53 ± 0.15
CPT-38	396.63 ± 0.22	353.59 ± 0.15
CPT-42	220.33 ± 0.15	190.33 ± 0.15
CPT-46	414.39 ± 0.15	361.88 ± 0.15
CPT-49	340.11 ± 0.15	310.55 ± 0.15
CPT-50	304.98 ± 0.15	263.88 ± 0.15
CPT-51	274.00 ± 0.15	154.69 ± 0.15
CPT-55	321.05 ± 0.15	220.33 ± 0.15
CPT-56	246.26 ± 0.15	176.64 ± 0.15
CPT-64	489.06 ± 0.15	413.08 ± 0.15
CPT-65	234.59 ± 0.15	222.14 ± 0.15
CPT-66	400.52 ± 0.22	355.92 ± 0.15
CPT-68	465.85 ± 0.22	395.27 ± 0.15
F Value	469441.2	588621.1
P Value	< 0.01*	< 0.01*

Values are given as Mean ± Standard Error (n=3); *denotes significant at 1% level

revealed 14.27. Lower the Ic_{50} value, higher is the antioxidant activity. From the results, it is clear that the sample CPT- 21 had higher activity than the other samples and was near the standard ascorbic acid. Thus, the inhibition activity was found to be dose- dependent, with a proportionate increase of DPPH along with concentration.

Characterization of Phytocompounds by GCMS:

The GCMS analysis was carried out in methanol extract of Tamarind pulp and the chromatogram had revealed 22 phytocompounds in which 8 compounds showed more than 1% peak area (Fig. 2). The compound 4h-pyran-4-one, 2,3-dihydro-3,5- dihydroxy – 6 - methyl showed 95.80%

Table 3: Quantitative analysis of flavonoids

	TOTAL FLAVONOID CONTENT (mg of Quercetin Equivalents/g of fresh weight)	
SAMPLE CODE	**AQUEOUS**	**METHANOL**
CPT- 3	151.88 ± 0.15	275.70 ± 0.15
CPT-7	83.633 ± 0.09	14.953 ± 0.15
CPT-19	211.38 ± 0.15	12.617 ± 0.15
CPT-21	183.24 ± 0.15	258.20 ± 0.15
CPT-23	175.07 ± 0.15	103.76 ± 0.15
CPT-25	166.62 ± 0.15	51.263 ± 0.15
CPT-35	130.88 ± 0.15	165.88 ± 0.13
CPT-37	122.87 ± 0.15	194.62 ± 0.15
CPT-38	120.38 ± 0.15	211.38 ± 0.15
CPT-42	116.74 ± 0.15	73.133 ± 0.15
CPT-46	108.87 ± 0.15	230.06 ± 0.15
CPT-49	103.76 ± 0.15	183.24 ± 0.15
CPT-50	99.677 ± 0.15	119.95 ± 0.13
CPT-51	99.237 ± 0.15	43.383 ± 0.15
CPT-55	94.857 ± 0.15	94.857 ± 0.15
CPT-56	93.117 ± 0.15	54.617 ± 0.15
CPT-64	350.37 ± 0.15	280.51 ± 0.15
CPT-65	69.487 ± 0.15	98.513 ± 0.13
CPT-66	61.763 ± 0.22	214.88 ± 0.15
CPT-68	58.263 ± 0.22	258.06 ± 0.15
F Value	233763.8	381900.7
P Value	< 0.01*	< 0.01*

Values are given as Mean ± Standard Error (n=3); *denotes significant at 1% level

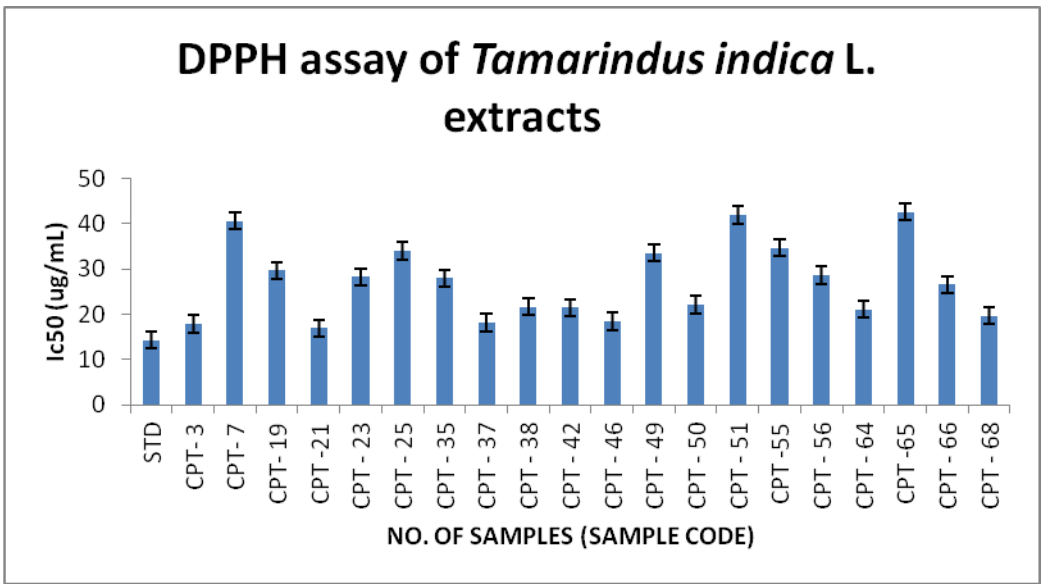

Fig. 1: DPPH assay of *Tamarindus indica* L. extracts.

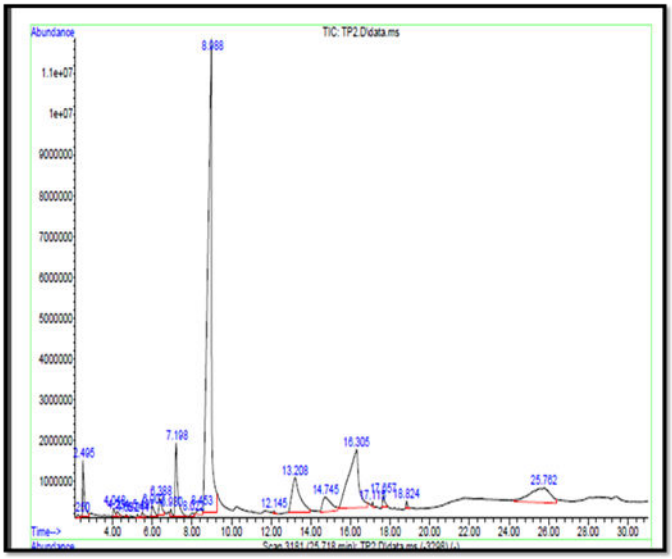

FIG. 2 :GCMS analysis of *Tamarindus indica* L. extract

Fig. 2: GCMS analysis of *Tamarindus indica* L. extracts.

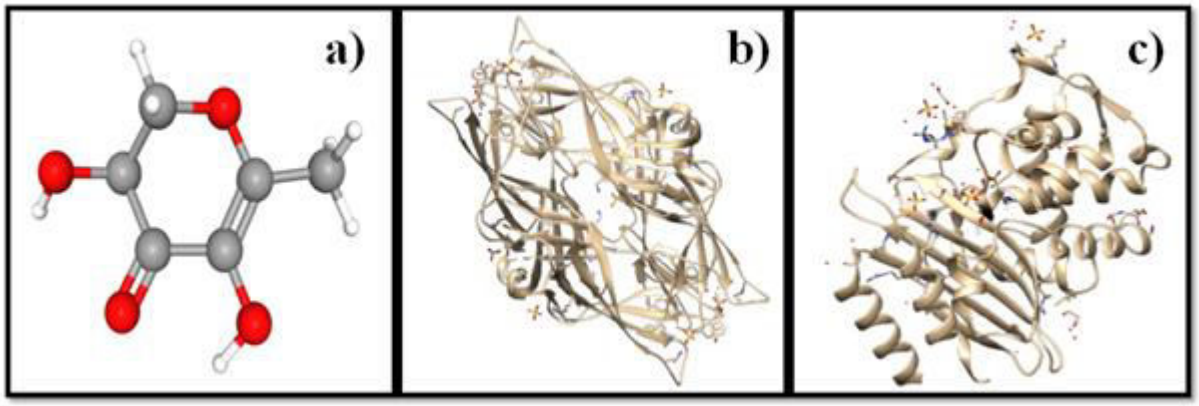

Fig. 3: 3D structure of the Ligand and target proteins. (a) Phytocompond Flavonol, (b) FmlH gene, (c) CTX-M beta lactamases.

probability when compared with the NIST library.

Recently, Flavonoids are becoming the subject of medical research because it possesses many biological properties like antimicrobial, antioxidant, anti inflammatory, enzyme inhibition and cytotoxic antitumor activity (Bhattacharya, 2011). The interaction of protein or enzyme with the flavonoid is due to presence of carbohydrate, phenyl ring, benzopyrone ring and phenol (Dana *et al*., 2021). Hence, the phytocompound 4h- pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6- methyl- a flavonol (Fig. 3) was selected as the potential ligand for molecular docking.

In Silico Docking Analysis:

Many uropathogens initiate a UTI using pili that mediate adhesion to host and environmental surfaces, facilitate invasion into the host tissues and promote interbacterial interactions to form biofilms. F9 pilus, is one of the most common pili in the *E. coli* genome and an important urovirulence factor employed by UPEC for the maintenance of UTI. FmlH, which is capable of binding terminal galactose (Gal), *N*-acetyl-galactosamine (GalNAc) within the inflamed bladder epithelium during chronic, unresolved UTI and Thomsen-Friedenreich antigen (TF)

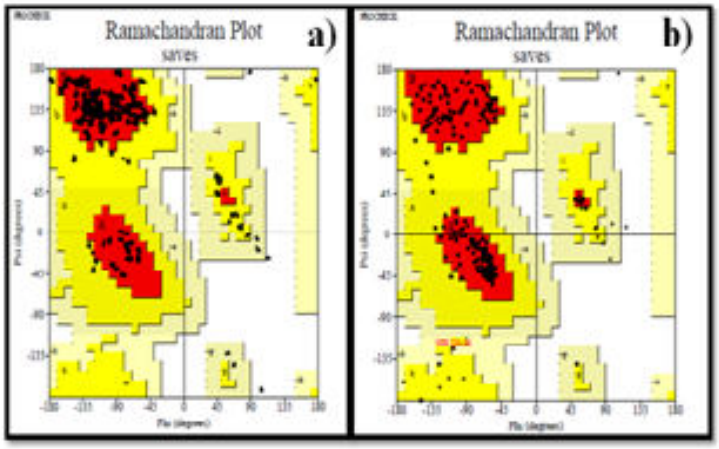

Fig. 4: Ramachandran plot of the target proteins molecule, (a) FmlH gene, (b) CTX-M beta lactamases.

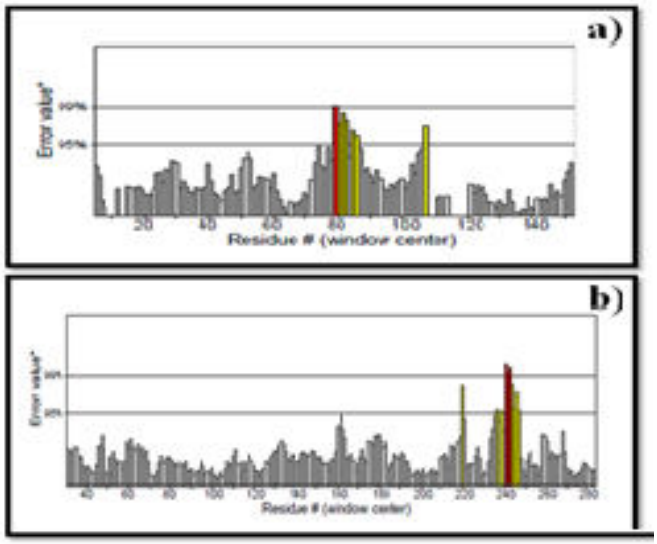

Fig. 5: EARRAT analysis of the target proteins molecule, (a) FmlH gene, (b) CTX-M beta lactamases.

within naive or infected kidneys (Vasilios Kalas *et al*., 2018). Thus, FmlH serves as a key role in the UPEC pathogenesis. Management of UTI has become problematic due to increasing production of extended-spectrum β-lactamases (ESBLs). Previously TEM and SHV enzymes were recognized as the main ESBLs. But recently, CTX-M has become more prominent and considered to be the most prevalent β-lactamases found in clinical isolates of *E. coli* globally. Cefotaximase-Munich (CTX-M) 15 is currently the most widely disseminated genotype associated with uropathogenesis and also the dominant multidrug resistant gene (Abdulaziz *et al*., 2017). Thus, FmlH and CTX-M 15 (Fig. 3) represent a promising target for molecular docking as anti virulence therapies for UTI in both the bladder and kidney.

Ramachandran plot was carried out for the validation of target protein molecules (FmlH gene and CTX- M-15) which had indicated the presence of 89.7% and 93.4% residues in most favoured regions (A, B, L). 10.3 and 6.1 per cent residues were found to be in additional allowed regions (a, b ,l, p), 0.4 per cent residues (CTX- M -15) were

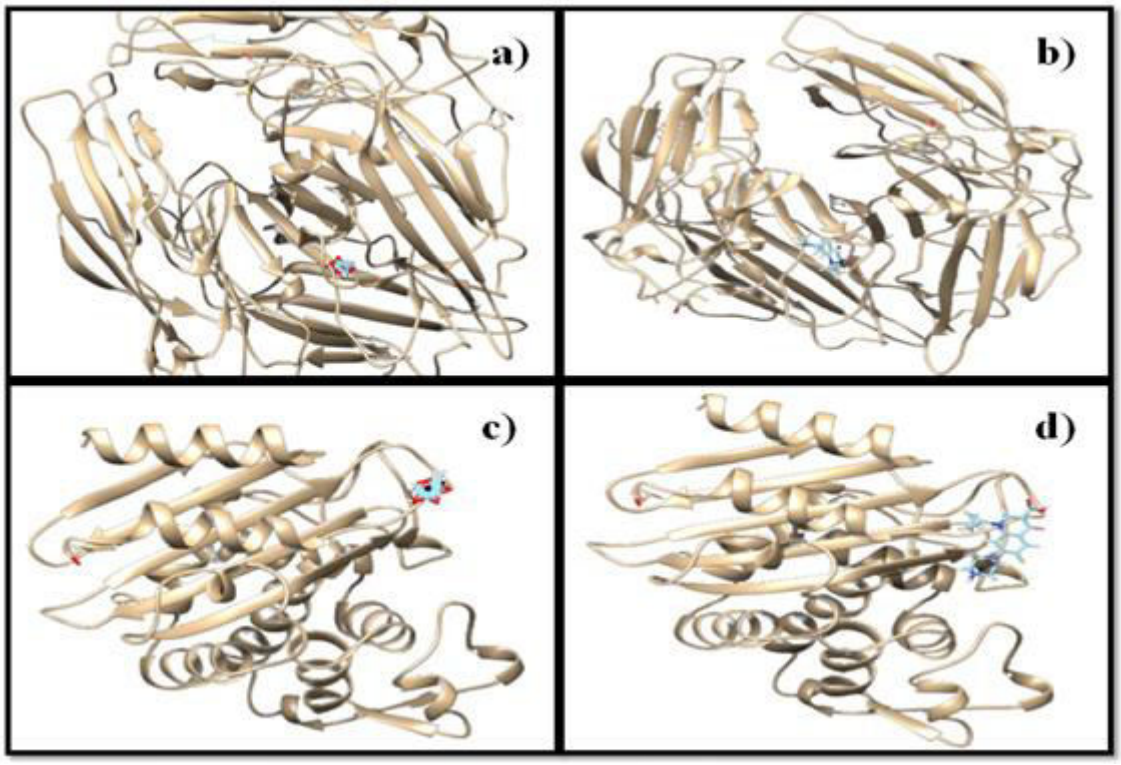

Fig. 6: Molecular Docking of Phytocompound and Standard Drug (Ciprofloxacin) with the target proteins (a) FmlH gene with Phyto compound, (b) FmlH gene with Ciprofloxacin, (c) CTX-M beta lactamases with Phytocompound, (d) CTX-M beta lactamases with Ciprofloxacin.

located in generously allowed regions (a,-b,-l,-p) and No residues (FmlH) were present in generously allowed regions [a,-b,-l,-p] and disallowed regions (Fig. 4). The Ramachandran plot is an indirect method for verifying that none of the geometries are located in the forbidden electrostatically unfavoured areas of the plot (Vaishali *et al*., 2019). Here, the results had revealed that no residues were found in the unfavoured region indicating that the selected protein structures are in good quality for molecular docking studies.

Non-bonded interaction between the various atoms in target protein molecules were evaluated by ERRAT analysis, where the errors are detected as random distributions of atoms in protein structures that can be distinguished from those displaying correct distributions (Colovos and Yeates, 1993). The results revealed an overall quality factor of 93.385 (FmlH) and 96.063 (CTX-M- 15) (Fig. 5).

At the atomic level, molecular docking can be used to model the interactions between proteins and small molecules, allowing us to study how small molecules behave at target proteins' binding sites while also elucidating fundamental biochemical processes (McConkey *et al*., 2002). Using DockThor, the phytocompound was docked against the targeted proteins. The docking process involves prediction of the ligand position, orientation of the sites and assessment of the binding affinity by docking scores (Xuan-Yu *et al*., 2011). With the scoring function, correct poses and binders can be easily separated from incorrect poses and inactive compounds in a reasonable computation of time (Kitchen *et al*., 2004). The docking score of flavonol against FmlH gene (Fig. 6) and CTX-M-15 (Fig. 6) was higher (-6.49 Kcal/mol and -6.62 Kcal/mol) when compared with the standard - ciprofloxacin (-7.32 Kcal/mol and -7.27 Kcal/mol). This revealed that the phytocompound was in good fit with the target

proteins in 3D space when compared with the standard drug which indicated the good binding efficacy and geometric complementarity score of the phytocompound.

The plant extract with antibacterial activities increasingly being found, more and more. Flavonoids with hydrophobic substituents such as prenyl groups are proven to be antibacterial agents (Rashid *et al*., 2013). Flavonols have been reported to be active agents against both gram-negative and gram-positive microorganisms where they generally exhibits better activity than the other class of flavonoids due to the presence of prenyl ring in their structure (Stermitz *et al*., 2002). Thus, the results have a clear evidence that the role of Flavonol in inhibiting the growth of uropathogenic organisms which may be due to the inhibition of nucleic acid synthesis, DNA gyrase, functioning and permeability of cytoplasmic membrane and the attachment of bacterial pili to the host tissues which in turn inhibit the formation of biofilms through interbacterial interactions by down- regulating the expression of adhesion genes (Cushnie and Lamb, 2005).

Conclusion

People use tomatoes for sour taste instead of tamarind because of the presence of organic acids. But tomato has a high level of uric acid which is one of the factors leading to UTI. Prevention or to reduce the possibility of UTI may be achieved by taking high amount of citrate, magnesium and carbohydrate. Naturally Tamarind pulp has these nutrients in addition to potential secondary metabolites which are suitable for the reduction of bacterial infection. Hence, an addition of appropriate amount of Tamarind pulp in our regular diet, having potential flavonoids with its synergistic effect of secondary metabolities, would definitely help in the prevention and management of uropathogenic bacteria leading to the occurrence of Urinary tract bacterial infections.

Acknowledgements

The authors thank the Head of the Department and Faculty members of Plant Biology and Plant Biotechnology of Ethiraj College for Women (Autonomous), Chennai for their valuable support and constant encouragement. We would also like to thank SRM University for extending facilities.

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