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GCMS VALIDATION OF LUTEOLIN FROM *APIUM GRAVEOLENS* AND *IN SILICO* DOCKING OF TLR-4 AND TLR-2

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Abstract

The flavonoid Luteolin, extracted from medicinal plants, has ability to cure hypertension, cancer and several inflammatory diseases. Gas chromatography mass spectrometry (GC-MS) is a major platform to analyze metabolic phenotypic studies of volatile and semi volatile molecules. This technique has several application in biomedical research such as drug screening, quality control and also other industrial applications. It is an efficient automated system that provides fast and effective results. GC-MS spectrum of the *Apium graveolens* ethanolic extract identified two major compounds Lucenin 2 (Molar mass 610.5 g/mol), derived from Luteolin and cis-11-eicosenamide with a mass of 309.5 g/mol. Docking has become one of the powerful tools in screening of therapeutic compounds, protein-protein interactions and in nano-research. Binding affinity of protein and ligands and their scoring functions could be well evaluated by docking. The membrane protein receptor, TLR-2 recognizes foreign substances by getting expressed on cell surface thereby passing appropriate signals to immune cells. Intracellular signaling pathway NF-κB and production of inflammatory cytokines are activated by TLR-4. These cytokines are responsible for activating innate immune system. Thus, stimulation of host immune response by TLR-2 and TLR-4 could be better option for treatment of various diseases. Hence, the present study is focused on Luteolin determination from a medicinal plant by spectrometry and also docking studies of Luteolin for the protein receptors TLR-2 and TLR-4.

Key words: Luteolin, *Apium graveolens*, TLR-2, TLR-4, GCMS, Docking.

Introduction

Luteolin is commonly present in plants and it is a form of yellow colour dye, utilized as a colouring source since several decades. It is a flavonoid of the flavone kind and is disseminated broadly in plants. Luteolin displays numerous activities like anti-inflammatory and anti-oxidant properties. Plants having Luteolin in high amounts are frequently utilized in medication for different infections for eg:- hypertension, tumors, and inflammation (Imran *et al.,* 2019; Juszczak *et al.,* 2019). Luteolin was named in 1829 by the French chemist Michel Eugene Chevreul and the right structure was proposed by an English chemist Arthur George Perkin in 1896.

Chromatography has an important role compared to other instrumental techniques in the examination of various compounds. It is used for quantitative identification and examination of the sample compound with high precision. It is basically a technique used for segregation and examining of mixtures of these compounds. Combining chromatography with other techniques involving analysis gives an exact detection and increase in analysing abilities, for complex mixtures of compounds (Szultka *et al.,* 2013; McNair and Miller, 2009). GC combined with mass spectrometry (GC-MS) gives a more accurate result (Majchrzak *et al.,* 2018) and the utilization of the technique in Luteolin determination is done following the standard procedure utilized for detection of flavonoids (Nolvachai and Marriott, 2013). Mass Spectrometry has been utilized for analysis of derivatives of Luteolin. (Schmidt *et al.,* 1993).

Toll-Like Receptor 4 (TLR4) is a part of the family of pattern recognition receptors (PRRs), receptors that perceive pathogen-associated molecular patterns (PAMPs) found in disease causing microorganisms and have a significant job in coordinating the advancement of an immune response against infectious agents (Kawai and Akira, 2010). TLRs are found in various infectious

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and noninfectious diseases, also in cancer and diseases related to immunity. They either advance or restrain the disease development (Molteni *et al.,* 2016; Marshak-Rothstein, 2006; Molteni *et al.,* 2006; Dajon *et al.,* 2017; Vijay-Kumar *et al.,* 2010). Systemic lupus erythematosus (SLE) is an autoimmune condition wherein toll like receptors 7 and 9 are involved in the antibody production against SLE (Marshak-Rothstein, 2006). The present study discusses on determination of Luteolin from *Apium graveolens* by gas chromatography mass spectrometry and also docking studies of the compound for the proteins TLR-4 and TLR-2.

Materials and Methods

Sample collection and extraction

Apium graveolens was dried and make powder; 100g of this dry powder was isolated with ethanol (500 ml) in a shaker for 48 hrs. Continuous isolation using the same solvent was done to get a colourless solvent. Then solution was evaporated to dryness and kept in a container.

GC-MS analysis

For the gas chromatography of plant extract, the Thermo DSQ II GC-MS was used. DSQ II has a column with DB 35ms. He gas was taken as carrier and with 1.0 ML/min of flow capacity. The oven temperature range maintained from 70°C to 260°C. The injector volume was 1ìl. The relative quantity of Luteolin present in the extracts of *A. graveolens* was expressed as percentage based on peak area produced in the chromatogram.

Protein- ligand Docking

The binding pattern and structure of Luteolin with TLR-2 and TLR-4 proteins were built in molecular modeling program (ChemSketch). The structure of TLR-2 and TLR-4 were retrieved from the Protein Data Bank (PDB). PDB is a database that contains the data of experimental structures of proteins & nucleic acids. These structures don't have information about bond orders, topologies etc.

The ligands were designed using ChemSketch and their 2D structure was converted to 3D structure using Chem 3D Ultra 6.1. to create or modify the chemical structures, the molecular modeling tool ChemSketch is commonly used and is one of the best tool for nuclear magnetic resonance spectrum and molecular property predictions, structure of chemical bond and functional groups handling software. For protein-ligand docking, the Gold software tool was used, this tool helps to understand the structure of binding and interactions. Genetic algorithm (GA) belongs to the evolutionary algorithm and mostly for gold software the GA is used to discover the flexible receptors including hydrogen bonds and flexibility of ligand. To identify the binding position of the ligand, Gold Score was used.

Results and Discussion

Apium graveolens Linn. (Karafs) is used in traditional medicine for the treatment of various ailments. The dried ripe fruit (sometimes called as seed) is mainly used for the medicinal purposes and commercially available in the market. For the conventional medicine preparation, celery has been used, mainly for the treatment of muscle pain and digestive issues and as diuretic, purgative, and calmative agents. In some African countries celery is taken for lowering the blood pressure (Lans CA., 2006). In the present study, GC-MS was used for the identification of Luteolin in *A. graveolens*, a combined form of two different analytical techniques and this is frequently used for identification and quantification purpose (Hites, 1997).

Fig. 1 shows the main chromatogram of the run sample and fig. 2 express the mass spectrum of the substance spotted at RT 32.16 min. The collected data from chromatogram were analyzed and compared with the details of compounds in GC-MS library database. Based on the vibrational stretches, the compound at the peak relative abundance at 149.1 m/z was identified as Lucenin 2 with molecular weight of 610.5 g/mol Fig. 3.

A total of 7 compounds were found in the sample. The active principles with their probability, molecular formula, molecular weight and area (%) of compounds found in the mass spectrum at 32.16 min retention time are presented in table 1. The top three major compounds found to be present in the ethanolic extract were Lucenin 2; 3, 20-dioxo-11-a-hydroxycoanine & 9, 12, 15-Octadecatrienoic acid with a percentage of 50.71, 12.15

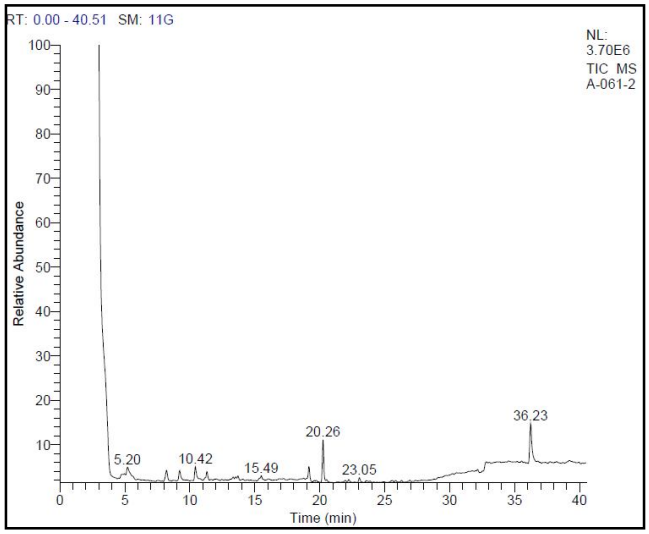

Fig. 1: Chromatogram of ethanolic extract of *A. graveolens*.

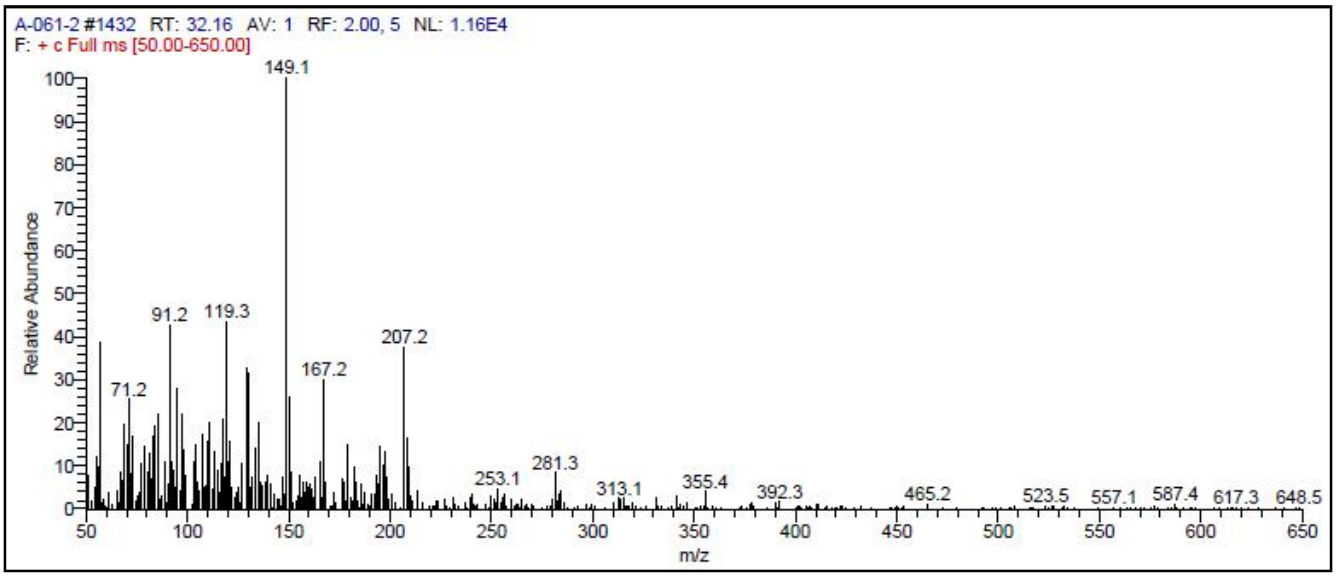

Fig. 2: Mass spectrum of the compound detected at RT 32.16 min.

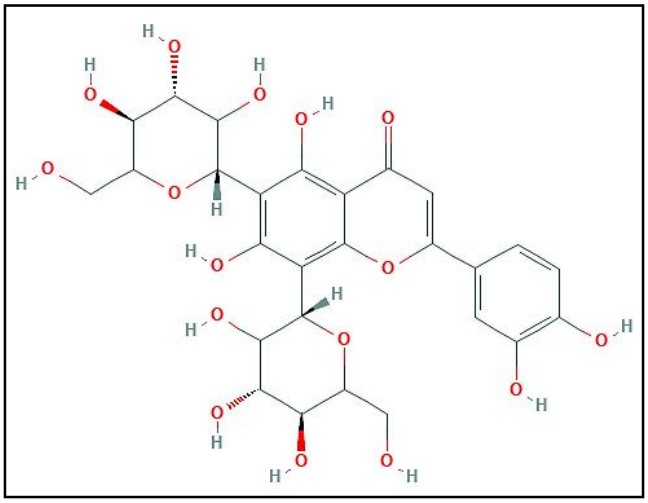

Fig. 3: Structure of Lucenin 2 with molecular formula $C_{27}H_{30}O_{16}$.

& 6.99 respectively. It is clear that Lucenin 2 is present in the highest amount in the ethanolic extract of *A. graveolens*.

Lucenin 2 is a C-glycosyl compound derived from Luteolin and was the predominant compound found in the extract. The molecular structure of Lucenin 2 is depicted in Fig. 3. Luteolin is a naturally occurring flavonoid with molecular mass of 610.5 g/mol that has potential anti-oxidant, anti-inflammatory and apoptosis inducing properties. These compounds are components that are found in human diet as they are constituents of plants. They have application in traditional medicine as therapeutic agents. Luteolin induces direct cell cycle arrest & apoptosis in tumor cells which inhibits tumor cell proliferation & suppresses metastasis (Zi, X *et al.,* 1998).

Fig. 4 shows the mass spectrum of the compound identified at RT 36.23 min. The active principles with probability, molecular formula, molecular weight & area (%) at RT 36.23 min is shown in table 2. The compound Cis-11-eicosenamide was found in the highest quantity with a percentage of 27.92 followed by 13-Docosenamide with a percentage of 7.46.

The constituents were identified by comparing GC-

Table 1: Chemical components at RT 32.16 min of ethanolic extract of *A. graveolens* matched with the GCMS library.

Compound Name	Proba-bility	Molecular Formula	Molecular Weight	Area %
Lucenin 2	50.71	$C_{27}H_{30}O_{16}$	610	1.41
3, 20- Dioxo -11- a-hydroxyconanine-1,4-diene	12.15	$C_{21}H_{27}NO_3$	341	1.41
9,12,15-Octadecatrienoic acid,2,3-bis[(trimethylsiyl)oxy] propyl ester,(Z,Z,Z)-	6.99	$C_{27}H_{52}O_4Si_2$	496	1.41
9, 12, 15-Octadecatrienoic acid,2,3-bis (trimethylsiyl)oxy propyl ester, (Z,Z,Z)-(CAS)	6.99	$C_{27}H_{52}O_4Si_2$	496	1.41
Methyl 7-Ethyl-10-Hydroxy-11-Hydroxy(180)-3, 11-Dimethyl-2, 6-Tridecadienoate	5.91	$C_{18}H_{32}O_4$	312	1.41
Benzoic acid, 4-methyl-[4-(methoxycarbonyl)phenyl]methyl ester (CAS)	4.52	$C_{17}H_{16}O_4$	284	1.41
Benzoic acid, 4-methyl-[4-(methoxycarbonyl)phenyl]methyl ester	4.52	$C_{17}H_{16}O_4$	284	1.41
Tricyclo [3.3.1.1(3,7)] decane-2,6-diol,2,5-bis(aminomethyl)-	1.92	$C_{12}H_{22}N_2O_2$	226	1.41
Tricyclo [3.3.1.1(3,7)] decane-2,6-diol,2,5-bis(aminomethyl)-	1.92	$C_{12}H_{22}N_2O_2$	226	1.41
1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS)	1.05	$C_{24}H_{38}O_4$	390	1.41

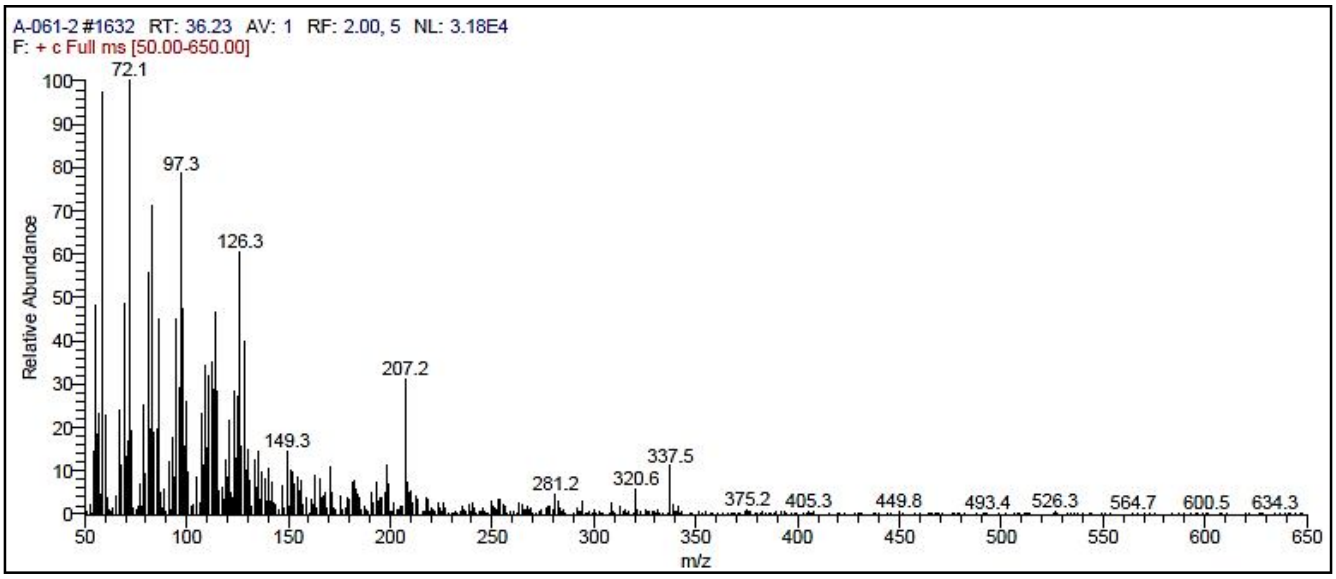

Fig. 4: RT 36.23 min, the mass spectrum of the compound identified.

Table 2: Chemical components at RT 36.23 min of ethanolic extract of *A. graveolens* matched with the GCMS library.

Name of the Compound	Compound Probability	Molecular Formula Compound	Molecular weight Compound	Area %
Cis-11-Eicoscenamide	27.92	$C_{20}H_{39}NO$	309	16.13
13- Docosenamide, (Z)-	7.46	$C_{22}H_{43}NO$	337	16.13
11- Octadecenal (spectrum disagrees) (CAS)	6.88	$C_{18}H_{34}O$	266	16.13
9-Octadecenamide, 12-hydroxy-,[R-(Z)]	6.61	$C_{18}H_{35}NO_2$	297	16.13
9-Octadecenamide	5.84	$C_{18}H_{35}NO$	281	16.13
9-Octadecenamide (CAS)	5.84	$C_{18}H_{35}NO$	281	16.13
9-Octadecenamide (Z)-	5.61	$C_{18}H_{35}NO$	281	16.13
9-Octadecenamide (Z)- (CAS)	5.61	$C_{18}H_{35}NO$	281	16.13
9-Octadecenoic acid, 1,2,3-propanetriyl ester(E,E,E)-	4.19	$C_{57}H_{104}O_6$	884	16.13
6-Octadecenoic acid	3.86	$C_{18}H_{34}O_2$	282	16.13

MS data with those given in library & reported in literature. Fig. 5 shows the molecular structure of the major compound Cis-11-eicosenamide at the peak relative abundance at 72.1 m/z with molecular weight of 309.5 g/mol. The non-enzymatic oxidation of Arachidonic acid function in various physical and morbid processes like: supporting or suppress different immune responses expressed by our body control the modulations in pregnancy and delivery; manage cell development; balancing human blood pressure; and regulating blood flow to whole cells (Edwards I.J., 2008).

The binding between proteins and ligand are predicted with the help of docking, is a computational technology. GOLD is a docking program for finding out the low energy docking modes at the active site of small molecules in protein binding. GOLD contains a group of programs for specific functions like for visualization and exploitation (Hermes), for predicting the position and direction of ligand in protein (GOLD) after processing (GoldMine). The Cambridge Crystallographic Data Centre (CCDC), UK

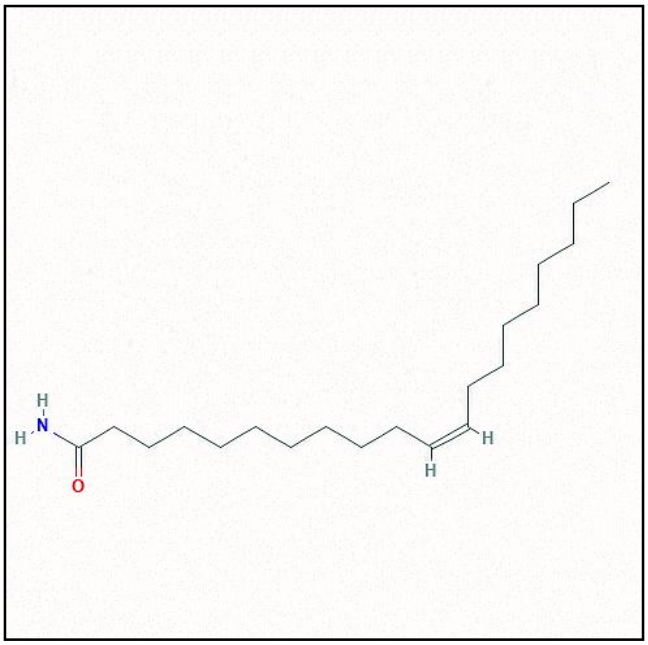

Fig. 5: Structure of cis-11-eicosenamide (Molecular weight: 309.5 g/mol, Molecular formula: $C_{20}H_{39}NO$).

developed the collaboration product, GOLD which is the mostly acceptable molecular modeling tool because of its accurateness and consistency. The docking results of Luteolin with the protein receptor in the present study showed the active site of toll-like receptor 2 (TLR-2) as ILE197, GLN198, LEU250, MET270, LEU282, ASN294, LEU312, PHE322, PHE325, TYR326, ASP327, LEU328, LEY33, LEU334, THR335, SER346, LYS347, VAL348, PHE349, LEU350, VAL351, PRO352, LEU355,SER356, TYR376, TRP386, PRO387, GLN390, ARG395, ASN414, SER421, LYS422, TYR440, ASN442, ASP463 and TYR483. Luteolin is docked to TLR-2 in these sites to form a complex. TLR-2 formed 6 bonds at the specific sites Fig. 6.

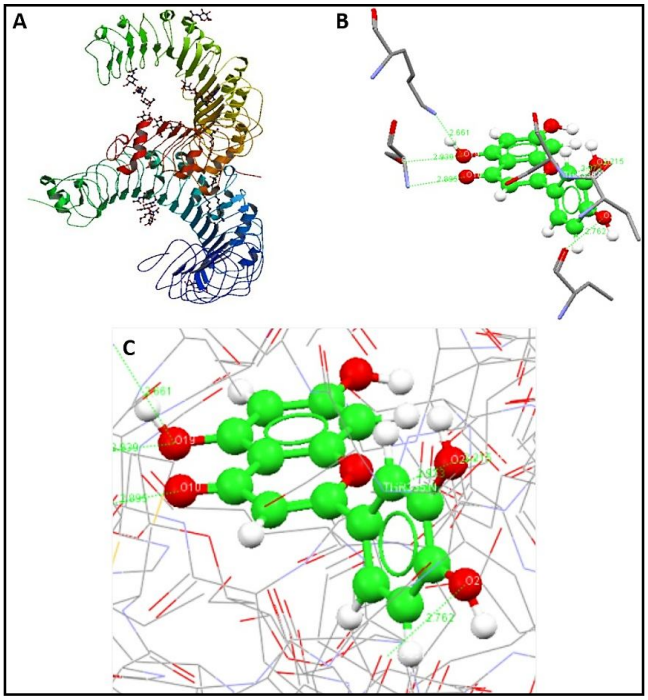

Fig. 6: Docking of Luteolin with TLR-2 protein. A. Structure of toll-like receptor 2 B. Luteolin in complex with toll-like receptor 2 C. Luteolin in complex with TLR-2.

Similarly, the active sites of toll-like receptor 4 (TLR-4) (PDB ID 4G8A) were identified as VAL 24, CYS 25, ILE 32, SER 33, TYR 34, ILE 46, VAL 48, CYS 51, ILE 52, GLU 53, LEU 54, SER 57, LEU 61, ILE 63, PHE 76, LEU 78, ILE 80, GLU 92, PHE 119, SER 120, PHE 121, ILE24, PHE 126, TYR 131, LYS 132, CYS 133, VAL 134, VAL 135, LEU 149, GLU 150, PHE 151, VAL 152, ILE 153. Luteolin is docked to TLR-4 at these sites to form a complex and formed 2 bonds at the specific sites Fig. 7.

Few applications of ChemSketch are available for free and when compared with other modeling tools, ChemSketch is highly accurate. The free applications include 2 dimensional structure cleaning tool, 3 dimensional

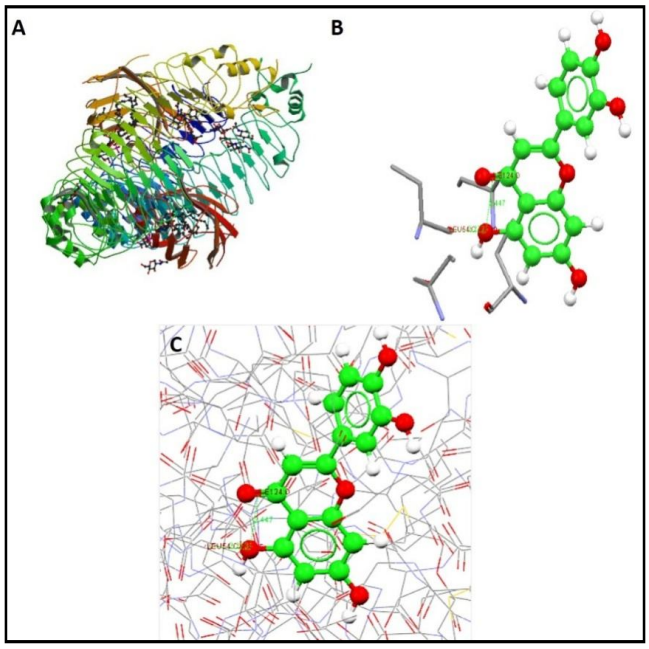

Fig. 7: Docking of Luteolin with TLR-2 protein. A. Structure Toll-like receptor 4 (TLR-4) B. Luteolin in complex with TLR-4 C. Luteolin in complex with TLR-4.

visualization and development. The table shows the receptors, ligand, protein and distance between bonds, the number of bonds and the score table 3. The lowest score has the best affinity to the ligand. TLR-4 has a score of 34.02 and TLR-2 has a score of 10.35 which implies TLR-2 has better affinity to Leuteolin.

Conclusion

Many natural compounds have been obtained from microbes and toll like receptors targeting plants. The low molecular weight compounds from natural sources have different cellular targets and anti-inflammatory effects, and only recognized to toll like receptor inhibition. The new targets of toll like receptors need deep study and efforts, this leads to the development of new modulators, they have the ability to manage the extreme inflammatory response at different medical conditions (Molteni *et al.,* 2018). This paper mainly discussed with the docking of

Table 3: Bond formation scores of ligand and protein by docking.

Receptors	Ligand	Protein	Distance b/w bonds	Score	No. of bonds
TOLL4	O9	ILE124:O	2.447	34.05	2
	O9	LEU54:O	2.273		
TOLL2	O21	LEU331:O	2.762	10.35	6
	O20	LEU334:O	2.25		
	O20	THR335:N	2.973		
	O19	TRP386:NE1	2.66		
	O19	SER356:OG	2.939		
	O0	SER356:N	2.895		

Luteolin with toll-like receptor 4 (TLR-4) and toll-like receptor 2 (TLR-2). There are thousands of chemical drawing tools available in market, Chem Sketch is one of the best tools and is most commonly used. Compared to other tools ChemSketch has some unique applications and easy to use, is an intelligent tool. Natural products from plants was combined with TL receptors to form new model for the synthesis of new drug or new compound. Here we have docked a protein TLR-4, 2 in human with a flavonoid extracted from the plant *A. graveolens* and the results showed better affinity of TLR-2 with Leuteolin. Here the flavonoid integrated with TLR-2 ,4 to form different complex models with separate active sites, as well as TLR 4 form 2 bonds and TLR 2 developed 6 bonds at specific sites.

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