Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

MOLECULAR CHARACTERIZATION, PHYTOCHEMICAL AND DOCKING STUDIES OF MOSQUITO REPELLENT COMPOUNDS IN *OCIMUM BASILICUM LINN.*VAR. *PILOSUM (WILLD.) - BENTH.*

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ABSTRACT

Many species of *Ocimum* reported to exhibit insecticidal and insect repellent properties. In the present investigation, molecular characterization of *Ocimum basilicum Linn. var. pilosum (willd)- Benth* was carried out with EST SSR markers and efficiently demarcated the *O.basilicum Linn. var. pilosum (willd)- Benth* from other *Ocimum* varieties. *The* leaf extract of *O.basilicum Linn. var. pilosum (willd)- Benth* was analyzed by GC-MS and 15 compounds were identified. Compounds which are structurally similar to the DEET were docked with Schrodinger mastero software. n-Hexa decanoic acid and , 4H-1-Benzopyran-4-One,5-Hydroxy-6,7-Dimethoxy-2-(4-Methoxyphenyl) were shown good docking scores with mosquito odorant binding protein 3N7H. N-Hexa decanoic acid present in *O.basilicum Linn. var. pilosum (willd)- Benth* found to be an ideal candidate for development of another potential mosquito repellent.

KEYWORDS: **EST SSR, Molecular Docking,** *O.basilicum Linn. var. pilosum (willd)- Benth*

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INTRODUCTION

Ocimum is the "mother medicine of nature" belongs to the family Lamiaceae and is grown in tropical and sub tropical regions in the world including India[1]. *Ocimum* has been attracted the attention of researchers due to its high contents of essential oil that is known to be insecticidal, larvicidal and mosquito repellent properties. These semiochemicals enter the olfactory systems of the insects and are transported across the lymph by Odarant Binding proteins (OBP) to the olfactory receptors on the olfactory receptor neurons (ORNs)[2]. Transfer of semiochemicals to ORNs induces the response in insects by transferring information to the central nervous system. The semiochemicals which are identified by the mosquitoes include Carbon dioxide, Lactic acid, Amino acid, Carboxylic acids, Phenols and many other compounds. These semiochemicals are identified to be involved in differential attraction of the insects to the hosts[3]. EST-SSRs are a valuable resource for the analysis of biodiversity and gene discovery. Bioinformatics based sequence analysis tools have extended the scope of ESTs into the fields of proteomics, marker development and genome annotation. The generation of ESTs facilitates gene discovery as they are direct representatives of the transcribable part of the genome and produced faster and inexpensive[4]. The essential oil composition and antimicrobial activity of different *Ocimum* species has been investigated in sufficiently large number of species globally[5,6,7]. However, essential oil composition in dried samples of *O.basilicum Linn. var. pilosum (willd)- Benth* has been reported[8,9]. Chemical constituents in the naturally available aromatic *Ocimum* species grown in fly ash dumping location typically exhibit novel oil composition and the results are contradicting to the earlier reports in the same species. It is thought that same species grown in different climatic conditions might alter the metabolism in production of essential oils in order to survive in the adverse environment and play a significant role in changeing the chemical composition. Gas chromatography-mass spectroscopy (GC-MS) is an analytical technique which includes two techniques, combined to form a single method of analyzing mixtures of chemicals. Gas chromatography separates the components of a mixture and mass spectroscopy characterizes each of the components individually. The present study primarily focused to determine the plant compounds that are similar to commercially approved mosquito repellent compound DEET. Further, studies have been conducted to analyse the potential ability to block the odorant receptor proteins of mosquitos which are well known to recognize semiochemicals from host and their key role in recognition of host seeking behavior through molecular docking or Insilco approaches from the plant compounds obtained by GC- MS studies of the plant extracts and carried out the characterization of compounds with EST-SSRs.

MATERIALS AND METHODS

Plant material

Plant material was collected from the Kondapalli Reserve Forest lies between Latitude.16.36°N, Longitude. 80.30°E at height about 168 meters above MSL in the Krishna district, Andhra Pradesh, India. *Ocimum* plant subspecies identification was done with the help of Botanical Survey of India, Southern Regional Centre, Coimbatore and specimen is maintained in the dept. of Biotechnology, KL University. Ayu, Kanchan, Vikar Sudha, Saumya varieties seeds were collected from CIMAP (Central Institute for Medicinal and Aromatic Plants, Hyderabad). All these plant seeds were successfully planted in the KL University herbal garden for further use.

EST SSR analysis

The EST sequences of *Ocimum* compounds were collected from the NCBI database. SSR sequences were identified with SSR IT software tool. Once SSR– containing ESTs were identified, flanking primers were designed using Primer 3 software[10]. Three primer pairs were synthesized and were used to detect the polymorphism in *O.basilicum Linn. var. pilosum (willd)- Benth*. PCR amplifications were carried out in a final volume of 10 µL PCR cocktail. The PCR

reactions were performed under standard conditions in a thermo cycler (Eppendorf). The annealing temperature was fixed for all primer pairs at 56°C. The PCR products were tested on 2 per cent agarose gel.

Table 1
SSR primer sequences

S.NO	Primer		Sequence	Bases
1.	KBG	FP	GCTACGATAGCACACCACCA	20
		RP	ATGCATGTCACACGTCGAT	19
2.	KGS	FP	GGAGGAAGCAAAATGGTTCA	20
		RP	TCTCCTCGCTCTTGCTCTTC	20
3.	KHARL	FP	GTCGTCCTCTGTTGGGATGT	20
		RP	AACAAGAAGCGGTCTCTCCA	20

KBG:2-hydroxy 6-methyl benzaldehyde, KGS: geraniol synthase, KHARL: Compound: N-hexadecanoic acid

DNA extraction and PCR analysis

About 2 g of young and healthy leaf tissue was collected from 30 day old plants and made into a fine powder in liquid nitrogen using a pre-chilled mortar-pestle. 50–100 mg of ground material was transferred into a 2 ml microcentrifuge tube containing 800 µl of pre heated CTAB buffer (Tris –HCL 75 mM, pH 8.0, EDTA 20 mM, NaCl 500 mM, CTAB 2% and PVP 1%) and added 1% β-mercaptoethanol to the centrifuge tube and incubated at 65˚C for 1hr. Later it was centrifuged at 13,000 rpm for 8min. and the supernatant transferred to a new microcentrifuge tube. To this, 240µl of phenol (pH 8.0) was added and then centrifuged for 3min and the top layer collected into a new microcentrifuge tube. To this supernatant, chloroform (24): iso amyl alcohol (1) mixture is added and centrifuged for 5 min. Without disturbing the interface, the supernatant was transferred to another 2 ml microcentrifuge tube, to this 100µl of 10M ammonium acetate and 1 ml absolute alcohol was added to the supernatant and mixed gently and incubated in ice bath for 10min. The tube was spun for 3 min in a microcentrifuge with maximum speed and the supernatant was decanted and then DNA pellet was dried and suspended in 200 µl of 1X TE (Tris-HCl 10 mM, pH 8.0, EDTA 1 mM) buffer containing 10 mg/ml of RNase A and then stored at -20^{0}C for long term storage or used directly for PCR amplification reactions. PCR cycling consisted of initial denaturation at 94^{0}C for 4 min, followed by 35 cycles of amplification of denaturation at 94^{0}C for 1 min, annealing at 55–65^{0}C for 1 min, and extention at 72^{0}C for 2 min. A final extension step at 72^{0}C for 7 min was followed by termination of the cycle at 4^{0}C. The amplified products (10 µl) were resolved on a 2 per cent agarose (Merck) gel.The amplification products were resolved on agarose gel (2%) and detected by Gel Documentation. The different bands obtained were evaluated by visual inspection, a 100 bp DNA ladder (New England Biolabs Ltd.) was used as a molecular weight marker. For the same primer, the products of different size were considered different alleles. The information obtained was coded in a worksheet for further analysis.

Collection and processing of leaves for Phytochemical study by using GC-MS

O.basilicum Linn. var. pilosum (willd)- Benth plant leaves were cleaned with deionized water and dried at shade for a week. The dried plant samples were ground well into a fine powder in a mixer grinder and sieved to give particle size of 50-150mm. About 25 grams of dried leaf powder samples were extracted with 200ml of the solvent in the temperature slightly above the boiling point (methanol -65°c), by hot percolation using a Soxhlet apparatus. The extractions were carried out for 7-8 hrs. Then the extracts were transferred into a beaker and the solvents were evaporated. The waxy residues obtained were up to 5ml. These crude extracts were stored in small glass screw cap tubes and were used for further analysis[11].

Analysis of active components using GC-MS

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas liquid chromatography and mass

spectrometry to identify different substances within a test sample[12]. GC-MS can provide meaningful information for components that are volatile, non-ionic and thermally stable and have relatively low molecular weight. The crude methanolic extracts of *O.basilicum Linn. var. pilosum (willd)- Benth* leaves were analyzed by GC-MS.GC analysis was performed using a SHIMADZU QP2010 equipped with a flame ionization detector and injector MS transfer line temperature of 240°C respectively. A Resteck-624 ms (30.0m x 0.32 mm, film thickness 1.8 (µm) was used. The oven temperature was held at 45 °C for 5 minutes and the temperature was raised, from 45-240°C at a rate of 2 °C /min. The carrier gas Helium (99.9995% purity) was maintained at a flow rate of 1.491 ml/min. One milliliter of extract mixed with methanol (80%), at a split ratio of 1:10 was injected. GC/MS analyses were carried out with GCMS solution ver.2.53 Software. Mass spectra were recorded ionization at (-70 eV). The scanning rate of 2 scans/sec and the run time was 35 minutes. Compound identification was accomplished by comparing the GC relative retention times and mass spectra to those of authentic substances analyzed under the same conditions by their retention indices (RI) and by comparison to reference components.

Identification of Compounds

The individual compounds identified from methanol extracts based on direct comparison of the retention times and their mass spectra with the spectra of compounds stored in the spectral database, NIST08s, WILEY8, and FAME libraries. (Table: 2)

Molecular Docking

3D structure of odorant binding protein of *Anopheles gambiae* (3N7H), was collected from PDB (Id:2ERB). Ligands were taken from various databases like ZINC, PubChem and sketched using tools like Chemsketch. While, selecting the ligand, the Lipinsky's Rule of 5 was applied. The rule is important for drug development where a pharmacologically active lead structure is optimized stepwise for increased activity and selectivity, as well as drug-like properties, as described. Glide calculations were performed with Schrodinger, Inc. It performs grid-based ligand docking with energetics and searches for favorable interactions between one or more typically small ligand molecules and a typical receptor molecule (usually a protein). After ensuring, the protein and ligands were in the correct form for docking and the receptor-grid files were generated using a grid-receptor generation program. A grid box was generated at the centroid of the active site amino acid residues[13] and the ligands to be docked were selected from workspace. The ligands were docked with the active site using the Glide standard docking procedure. Then the ligands with best Glide score were subjected to precision docking algorithm. Glide generates conformation internally and passes through a series of filters. In the first filter stage, at various grid positions($1A^0$) the ligand center places turn around the three Euler angle. Later the crude score values and geometric filters remove improbable binding modes. In the subsequent filter stage, a grid based force field evaluation and refinement of torsional and rigid body movements of the ligand were carried out with the help of OPLS-AA forced field. Finally, Monte Carlo method was adapted for minimization of energy score of the remaining small number of surviving docking solutions[14]. The final energy evaluation is done with Glide score and a single best pose is generated as the output for a particular ligand.

$$G\ Score = a \times vdW + b \times Coul + Lipo + Hbond + Metal + BuryP + RotB + Site$$

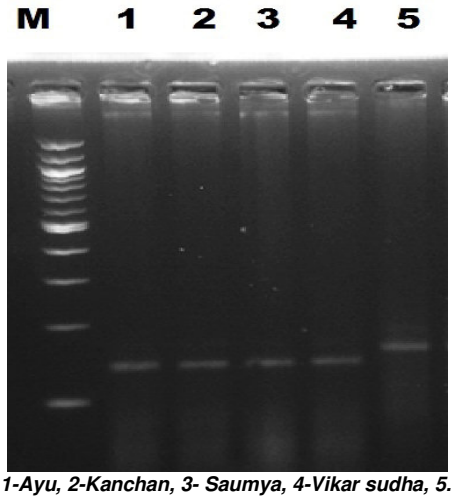
where, vdW => van der Waal energy; Coul => Coulomb energy; Lipo => lipophilic contact term; HBond => hydrogen-bonding term; Metal => metal-binding term; BuryP => penalty for buried polar groups; RotB => penalty for freezing rotatable bonds; Site => polar interactions at the active site; and the coeffi cients of vdW and Coul are: a = 0.065, b = 0.130.

RESULTS

Molecular characterization with EST-SSR markers

EST SSRs were successfully validated in five *ocimum* varieties *viz.,* Ayu, Kanchan, Saumya, Vikar Sudha and *O.basilicum Linn. var. pilosum (willd)- Benth*. PCR amplified products were separated on 2 per cent agarose gel. Among these primers, KHARL clearly differentiated the *O.basilicum Linn. var. pilosum (willd)- Benth* with amplification size of 130 bp (Lane 5) from other *ocimum* varieties typically exhibit amplicon size of125 bp (Lane 1 to 4). PCR products amplified with KHARL was shown in Figure 1. The primers KBG and KGS were amplified at 250 bp and 160 bp respectively. But no polymorphism was observed among tested *Ocimum* varieties.

Figure 1
EST -SSR profile of KHARL Series of Markers



M=Marker 1-Ayu, 2-Kanchan, 3- Saumya, 4-Vikar sudha, 5. O. pilosum

GC- MS Results

O.basilicum Linn. var. pilosum (willd)- Benth essential oils were isolated from the leaves and the chemical compounds were analyzed with Gas Chromatography and Mass Spectrophotometer. Chromatogram showing the retention time and retention peaks of different compounds of *O.basilicum Linn. var. pilosum (willd)- Benth* were shown in Fig.2. The qualitative and quantitative compositions of the leaves of *O.basilicum Linn. var. pilosum (willd)- Benth* were shown in (Table .2). Cis-9-Hexadecenal shows highest peak and its retention time is 18.20 with 35.06% of area, followed by n-Hexadecanoic acid, its retention time is 16.509 with 21.60% of area and next followed by 2-Hydroxy-6-Methylbenzaldehyde, its retention time is 10.847 with 10.99% of area less than 8% 4H-1-Benzopyran-4-One,5-Hydroxy-6,7-Dimeth oxy-2-(4-Methoxyphenyl)–(7.75%,26.087), Phytol (4.37%, 17.88), Cycloisolongifolene, 7-bromo-(3.31, 17.91), Neophytadiene (2.75%, 15.15), Benzoic acid (2.62%, 7.09), Olealdehyde (2.40%, 21.04), 1,2,3,-propanetriol, monoacetate (2.16%, 8.15), Geranic acid (1.92%, 9.50), p-Methyl benzoic acid (1.73%, 8.53) 1,2-Benzenediol (1.40%, 7.57), All-trans-squalene (1.24%, 23.50), Propylure (0.69%, 10.1).

All of these compounds molecular formula were preseted in table 2.

Figure 2
Chromotogram of O.basilicum Linn. var. pilosum (willd)- Benth leaf extract

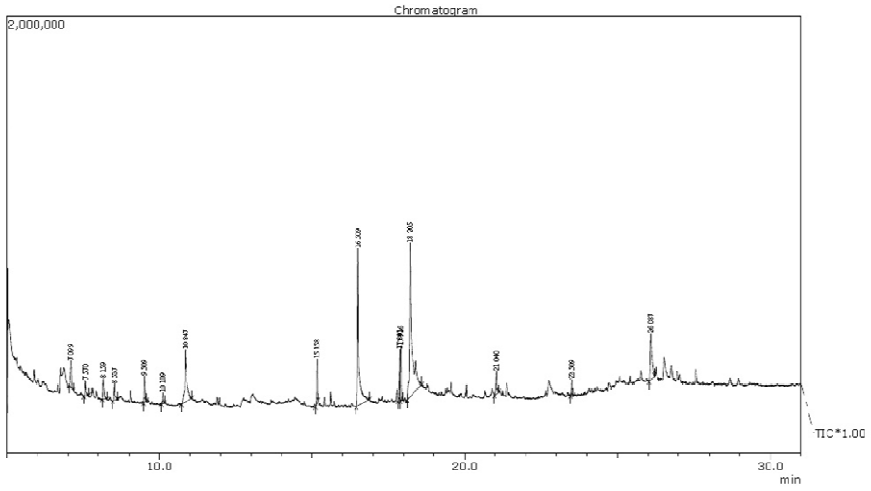


Table 2
Different compounds of O.basilicum Linn. var. pilosum (willd)- Benth obtained by GC-MS analysis

Peak.No	Ligand Name	Molecular formula	Area %	R.Time	G-Score	Lipophilic EvdW	H-Bond
1	benzoic acid	C_6H_5COOH	2.62	7.099	-5.14	-0.4	-0.35
2	1,2-Benzenediol	$C_6H_6O_2$	1.40	7.570	-3.49	-0.03	-0.96
3	1,2,3-Propanetriol, monoacetate	$C_5H_{10}O_4$	2.16	8.159	-3.66	-0.02	-1.15
4	p-Methylbenzoic acid	$C_8H_8O_2$	1.73	8.537	-5.15	-0.41	-0.35
5	Geranic Acid	$C_{10}H_{16}O_2$	1.92	9.509	-5.72	-1.21	-0.35
6	Propylure	$C_{18}H_{32}O_2$	0.69	10.109	-3.89	-1.93	-0.7
7	2-hydroxy-6-methylbenzaldehyde	$C_8H_8O_2$	10.99	10.847	-3.15	-0.42	-0.91
8	neophytadiene	$C_{20}H_{38}$	2.75	15.158	-1.41	-3.32	0
9	**n-Hexadecanoic acid**	**$C_{16}H_{32}O_2$**	**21.60**	**16.509**	**-7.3**	**-2.53**	**-0.35**
10	phytol	$C_{20}H_{40}O$	4.37	17.881	-5	-2.92	-0.35
11	Cycloisolongifolene, 7-bromo-	$C_{15}H_{23}Br$	3.31	17.916	--	--	0
12	cis-9-Hexadecenal	$C_{16}H_{30}O$	35.06	18.205	-4.31	-2.99	0
13	Olealdehyde	$C_{18}H_{34}O$	2.40	21.040	-4.18	-2.95	0
14	Squalene	$C_{30}H_{50}$	1.24	23.509	-3.68	-3.94	0
15	**4H-1-Benzopyran-4-one,5-Hydroxy-6,7-Dimethoxy-2(4-Methoxyphenyl)**	**$C_{18}H_{16}O_6$**	**7.75**	**26.087**	**-7.0**	**4'-trimethoxy flavone**	**-0.63**
16	**DEET**	$C_{12}H_{17}NO$	**----**	**----**	**-2.74**	**-0.48**	**-0.7**

Molecular Docking

The leaf extract compounds which are structurally similar to the DEET were docked with mosquito odorant binding protein 3N7H. This receptor active site region was confirmed using Schrodinger Computer Aided drug Design Software (Scrodinger, Inc.). DEET was used as a control for molecular docking with Glide score (-2.74), as shown in Table 2. The results clearly established high binding affinities of n-Hexa decanoic acid and 4H-1-Benzopyran-4-One5-Hydroxy-6,7-Dimethoxy-2-(4-Methoxyphenyl)- with glide scores of -7.3 and 7.0 respectively (Table 2). The

interactions of DEET, Palmitic acid and 4H-1-Benzopyran-4-One, 5-Hydroxy-6,7-Dimethoxy-2-(4-Methoxyphenyl)- with 3N7H protein, through the amino acid residues of 3N7H and the hydrophobic binding pocket surrounding DEET, Palmitic acid and 4H-1-Benzopyran-4-One, 5-Hydroxy-6,7-Dimethoxy-2-(4-Methoxyphenyl)- confirmed the affinity (Figures 3-7).

Figure: 3 Structure of 3N7H crystal of OBP.

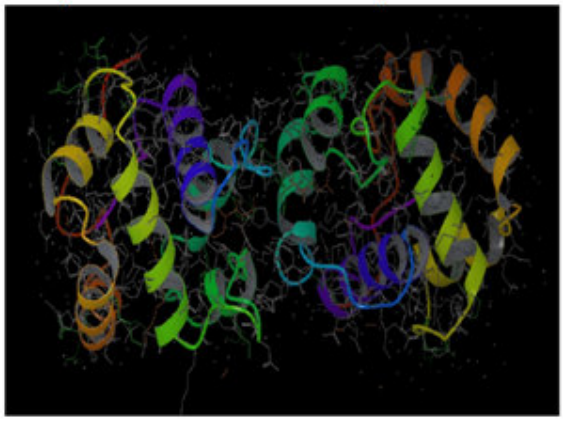
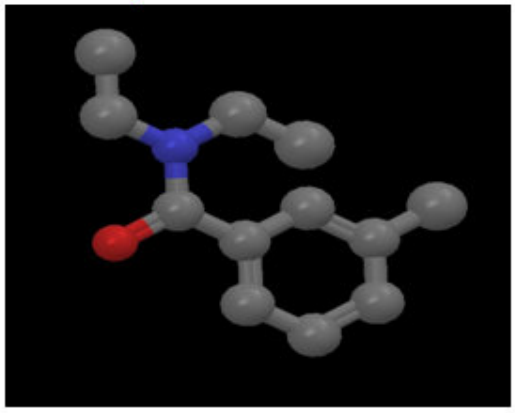


Figure:4 Structure of DEET



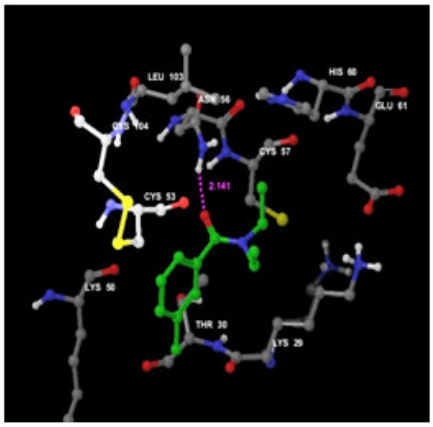


Figure 5
Docking of DEET with 3N7H

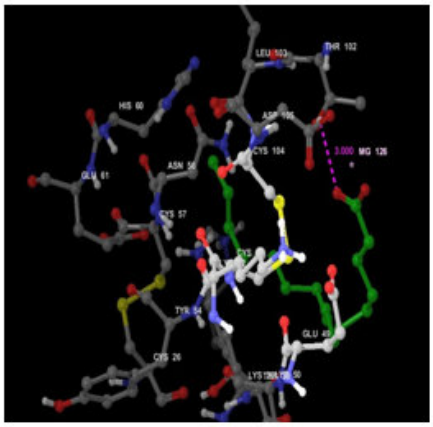


Figure 6
Docking of n-Hexadecanoic acid with 3N7H

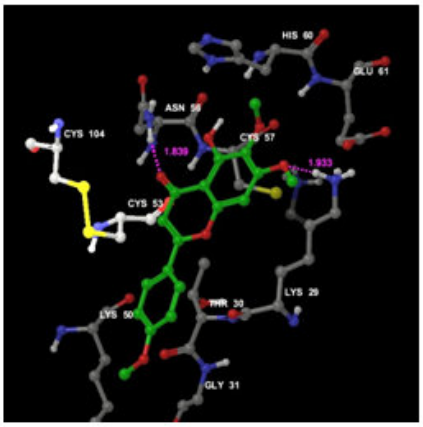


Figure 7
Docking 4H-1- Benzopyran -One, 5-Hydroxy-6,7-Dimethoxy -2-(4-Methoxyphenyl)-with 3N7H

In the present investigation, three EST SSR primers were designed using primer 3 software tool. These three EST SSR markers were evaluated in *O.basilicum Linn. var. pilosum (willd)- Benth* along with four *Ocimum* varieties. Out of three markers, KHARL marker was shown distinct polymorphism between *O.basilicum Linn. var. pilosum (willd)- Benth* and other four *Ocimum* varieties. Based on this result, KHARL marker can be used for DNA finger printing in *O.basilicum Linn. var. pilosum (willd)- Benth*. Variations in the DNA sequence have been used as molecular markers in plants[15]. Genetic relationships in *Ocimum* species using molecular markar banding data may be useful for plant improvement and an efficient way to conserve genetic resources of *Ocimum* species, in addition to their effective medicinal uses[16]. DEET is a synthetic mosquito repellent is considered as reference ligand for this molecular docking study[17]. All compounds with major area percent obtained from the GC-MS analysis of *O.basilicum Linn. var. pilosum (willd)- Benth* were docked with the odorant binding protein 3N7H. Novel active compounds from plant origin, and to assess the efficient therapeutic properties with minimum side effects, application of advanced methods like GC MS and computational techniques play a crucial role in designing and development of drug of interest[18]. Molecular

docking results based on the G-score, H-Bond and residue interaction shows binding affinity of the ligands towards protein 3N7H. If Glide score is more, the binding affinity of the ligand is higher. The G-Score of DEET is -2.74, when compared with the compounds of *O.basilicum Linn. var. pilosum (willd)- Benth.* G-Score of n-Hexa decanoic acid is -7.3 and 4H-1-Benzopyran-4-One, 5-Hydroxy-6,7-Dimethoxy-2-(4-Methoxyphenyl) is 7.0, which is greater when compared to DEET potential option to design and develop novel repellents to fight against pathogenic mosqitoes .

ACKNOWLEDGEMENT

Authors thank Dr.T.Venkateswara Rao, for providing the Schrodinger mastero software and technical discussions in preparation of the manuscript and Sargam Laboratories for GC-MS analysis. Authors especially thank to Department of Biotechnology, KL University for providing financial assistance and lab facilities for successful completion of the present work.

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