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KARNATAK UNIVERSITY DHARWAD

OMICS International

Global Summit on Herbals and Natural Remedies

"Unravelling the medicinal potencies of unexploited species of Gentianaceae family – *Exacum pedunculatum*"

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Introduction

Natural products and medicine have been closely linked from the ancient times through the traditional practices.

Scientific developments have allowed progress in understanding the mechanism of action of the traditional medicines.

Although most herbal medicines have a long history of traditional use, only their experimental validation gives a clear idea about its safety and efficacy.

Importance

The role of classical medicines are well documented and appreciated throughout the world which are sourced from traditional medicines. The usage of medicinal plants is traditionally rooted as an essential part of public healthcare in India.

Diabetes is a major health problem with complicated metabolic disorder that has gravely troubled the human health and quality of life. Though there are various approaches to reduce ill effects of diabetes and its secondary complications, herbal formulations are preferred due to less side effects and low cost.

The study focuses on the plant which is effectively anti-diabetic in nature and aiming to establish mechanism of action.

Plant Description

Exacum pedunculatum is phytochemically unexplored, traditional medicinal herb. Because of its bitter taste, local people used it as a remedy for diabetes and skin diseases. Present study provides a thorough assessment of the profile of the plant to ensure the rational use in diabetes management.

The plant was collected from Western Ghats of Karnataka and identified in the herbarium of Department of Biotechnology and Microbiology. The voucher specimen (MNHN-P-P00488786) is deposited in the dept of Biotechnology , Karnatak University, India.

Taxonomic Classification :

Kingdom – Plantae
Phylum – Magnoliophyta
Class – Magnoliopsida
Order – Gentianales
Family – Gentianaceae
Genus – Exacum
Species – *Exacum pedunculatum*

Exacum pedunculatum

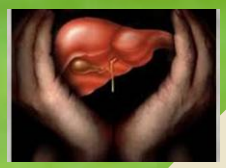
Plant Description -

Exacum pedunculatum commonly known as stalked Persian violet is a small annual herb, 7-30 cm tall, with four angled stem. Oppositely arranged leaves are stalkless, lanceshaped, 3-5 cm long, 2-5 nerved, with tapering base. Flowers are borne in branched cymes. The stalks carrying the cymes and the flowers are long and rigid. Flowers are purple or blue, with 4 lanceshaped petals. Sepals are 4, winged. Stamens are 4, with short filaments. Capsules are round, 4 mm, shining brown. Flowering August to September.

Collection of Plant Material -

Fresh plant material was collected in the rainy season from the grasslands, in the plains to Low Altitude and Moist Localities of the Western ghats of Kemmangundi, Chikamagalur district, Karnataka, once during early September 2014 and late October 2014. The aerial parts of the plant was shade dried, powdered mechanically, and stored in moisture free containers.

Overview

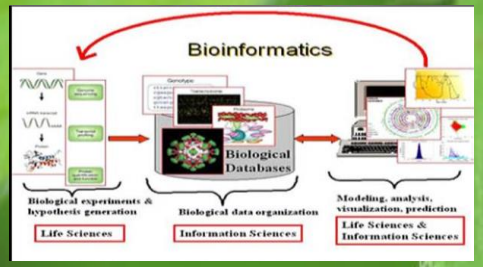


Protection

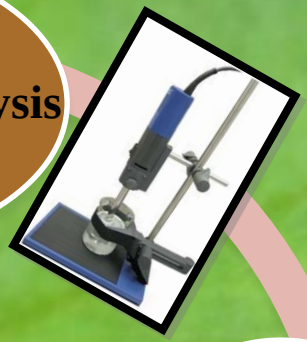
Target Prediction, visualization

Informatio n Science

Life Science



Phytochemical Analysis

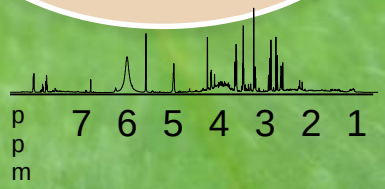


Functional Enrichment

Traditional knowledge

Isolation & Purification of the compound

Chemical & Data Analysis



Diabetes..

Both T1DM and T2DM are chronic conditions that typically cannot be cured, however can be treated since the development of insulin in 1921.

The enhancement of insulin secretion by pancreatic β-cells is a major goal for the treatment of T2DM. Antidiabetic drugs or hypoglycemic agents lower blood glucose levels.

They basically work on

- **Increasing insulin levels in the body.**
- **Increasing the body's sensitivity to insulin.**
- **Decreasing glucose absorption in the intestine.**

Classes of drugs and their side effects (Few examples)

There are different molecular targets with their own mechanism of action but are also associated with side effects which ends up with different complications, thus affecting the quality of life.

For ex: α-glucosidase inhibitors, (acarbose, miglitol), are effective at decreasing the absorption of glucose by intestine are often associated with abdominal bloating, diarrhoea and flatulence.

Conventional insulin secretagogues, such as sulfonylureas and meglitinides, both result in the induction of hypoglycemia.

While metformin is the only therapuetic agent that has been demonstrated to reduce macrovascular events in T2DM, it is not recommended in patients who have decreased renal or hepatic function.

And also hypoglycemia and weight gain are the common side effects. Thus, new approaches are needed to treat T2DM.

The molecular target -

- There are different drugs that act on different targets for the treatment.
- The choice of the target in the studies is Peroxisome-proliferator activated receptor (PPAR γ)
- They are the members of nuclear receptor superfamily that regulate the gene expression of proteins involved in energy, glucose and lipid metabolism, the proliferation and differentiation of adipocytes and the sensitivity of insulin.
- They function as cellular sensors that activate transcription in response to the binding of natural or synthetic ligands.
- Three cellular subtypes PPARα, PPARβ/δ and PPARγ, have been identified. Though they share high level of sequence and structural homology, they exhibit differences in expression and physiological functions.

Contd...

- PPARα is found in the liver, kidney, heart, and muscle. It helps in the uptake and oxidation of fatty acids and lipoprotein metabolism. PPARα is the target of lipid lowering fibrates.

- PPARγ is localized in fat, large intestine, and macrophages. It plays an important role in adipocyte differentiation.

- **Agonists of PPARα and PPARγ are currently approved for use in treating dyslipidemia and T2DM, respectively .**

- PPAR β/δ is expressed in most cell types. PPARβ/δ agonists play important roles in dyslipidemia, cancer treatment, and cell differentiation within the central nervous system.

Functioning of PPAR..

PPARs function through the formation of heterodimers with the retinoid X receptor (RXR) and dock to the promoter regions of genes, which regulates transcription in a ligand-dependent manner through the differential recruitment of co-activators and co-repressors .

PPARγ can be considered a rheostat for insulin sensitivity that responds to an integrated nutritional status conveyed through multiple signals sensitive to the dietary and endocrine status .

Initial Experiments

Preparation of the Plant extract -

- The powdered plant material was subjected to hot extraction in Soxhlet apparatus using petroleum ether as solvent.
- The extract was evaporated to dryness under reduced pressure using a Rotavapor.
- Extract was stored in airtight tubes and a portion of it was used for phytochemical analysis and structural studies.

Preliminary Phytochemical investigation -

Phytochemical tests were employed in the preliminary screening for various secondary metabolites such as alkaloids, glycosides, saponins, flavonoids and tannins.

Table 1: Phytochemical screening of the Petroleum ether extract of *Exacum pedunculatum*

Test	Observation	Inference
1) Test for alkaloids		
a) Mayer's test (potassium mercuric iodide) To a few drops of the Mayer's reagent, 2 mg of extract was added.	Formation of white or pale yellow precipitation, indicated the presence of alkaloids.	**Positive**
b) Wagner's test (solution of iodine in potassium iodide) 2 mg of extract was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent were added.	A yellow or brown precipitation indicates the presence of alkaloids.	**Positive**
2) Test for Flavonoids		
a) Ferric chloride test Test solution (small quantity of extract +2 ml of water) in a test tube and a few drops of ferric chloride solution was added.	Intense green colour was appeared	**Positive**
b) Alkaline reagent test Test solution (small quantity of extract +2 ml of water) in a test tube sodium hydroxide solution was added.	Solution mixture shows increase in the intensity of yellow color which becomes colourless on addition of few drops of dilute acids.	**Positive**
c. Shinoda's Test In a test tube containing 0.5 ml of the extract 10 drops of dilute hydrochloric acid followed by a small piece of magnesium were added.	Formation of pink, reddish or brown colour indicates the presence of flavonoids.	**Positive**

Contd...

3) Test for Glycosides **a) Keller-killiani test** To the test solution (small quantity of extract) few drops of ferric chloride solution was added and mixed well. Then concentrated sulphuric acid was added slowly two layers are formed.	The upper layer was bluish green coloured and lower layer was reddish brown coloured.	**Positive**
4) Test for saponins **a) Foam test** Small quantity of extract was treated with 5 ml of water and shaked well.	It shows formation of froth which was found to be stable for about 15 min.	**Negative**

Contd...

5) Test for steroids **a) Salkowaski test** 2 mg of extract was shaken with chloroform, to the chloroform layer sulphuric acid was added slowly by the sides of the test tube. **b) Lieberman buchard test** 2 mg of extracts was dissolved in acetic anhydride, heated to boiling , cooled and then 1 ml of concentrated sulphuric acid added along the sides of the test tube.	Formation of red colour indicates the presence of steroids**.** A brown ring was formed at the junction of the two liquids and the upper layer colour turned to green.	**Positive** **Positive**
6) Test for tannins **a) Ferric chloride test** 0.2 gm of extract was boiled in 5 ml of water. The mixture was cooled and filtered. A few drop of 5% ferric chloride solution was added to the filtrate. **b) Gelatin test** Test solution (small quantity of extract) taken in a test tube, was treated with gelatin solution,	Formation of Blue-Black precipitation White precipitate.	**Negative** **Negative**

Structural analysis of major components -

(i). Thin Layer Chromatography -

Crude extract was dissolved in petroleum ether and spotted on TLC strips . The strips were developed in chromatography chamber containing solvent mixture of petroleum ether, ethyl acetate and hexane in different concentrations. the developed strips were air dried and visualized under UV light.

Observation-

TLC Bands Developed -

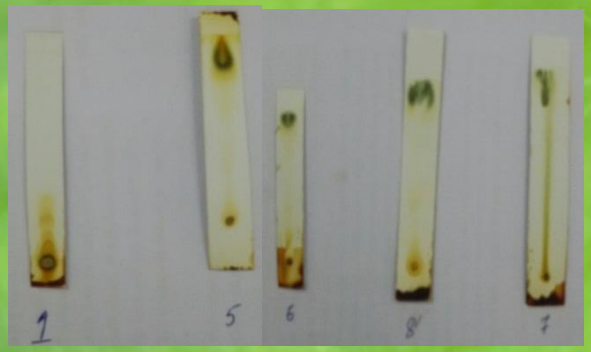
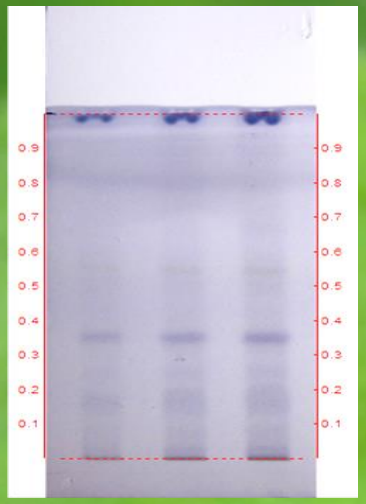


Fig 1: Separation of compouds of petroleum ether extract *of Exacum pedunculatum* by TLC method

Developing Solvent	**Observation**	**Spot(s)**	**R_f Value**
Petroleum ether	Naked eye, UV, Iodine Vapours	2	0.125 Yellow 0.2Light Brown
Ethyl acetate:Pet ether (80:20)		3	0.7 Yellow 0.8 Green 0.9 Brown
Ethyl acetate:Pet ether (50:50)		1	0.75 Green
Ethyl acetate		1	0.75 Green
Ethyl acetate:Pet ether (90:10)		1	0.75 Green

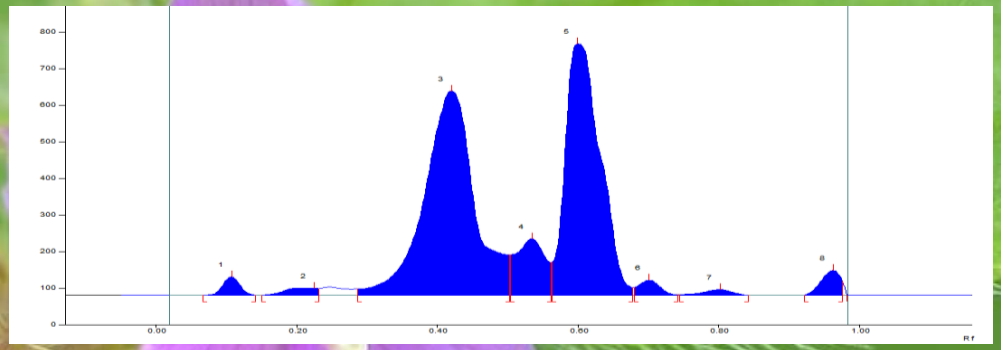
Table 2: Retention values of the crude extract

High Performance Thin Layer Chromatography



Exacum pedungulatum -Pet Ether extract at 4µl; 2-8µl; 3-12µl

Solvent system: Toluene : Ethyl Acetate (9:1)



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.07 Rf	0.1 AU	0.11 Rf	50.3 AU	3.16 %	0.14 Rf	0.1 AU	881.3 AU	1.42 %
2	0.15 Rf	0.1 AU	0.23 Rf	19.0 AU	1.20 %	0.23 Rf	18.4 AU	649.3 AU	1.05 %
3	0.29 Rf	16.9 AU	0.42 Rf	557.1 AU	34.99 %	0.50 Rf	10.5 AU	28432.4 AU	45.96 %
4	0.50 Rf	110.6 AU	0.54 Rf	154.5 AU	9.71 %	0.56 Rf	89.0 AU	4612.6 AU	7.46 %
5	0.56 Rf	90.3 AU	0.60 Rf	686.4 AU	43.12 %	0.68 Rf	21.4 AU	24597.9 AU	39.76 %
6	0.68 Rf	21.6 AU	0.70 Rf	41.2 AU	2.58 %	0.74 Rf	1.6 AU	906.6 AU	1.47 %
7	0.75 Rf	1.6 AU	0.80 Rf	15.6 AU	0.98 %	0.84 Rf	0.1 AU	464.2 AU	0.75 %
8	0.92 Rf	0.3 AU	0.96 Rf	67.9 AU	4.27 %	0.98 Rf	38.2 AU	1323.7 AU	2.14 %

Fig2. *Exacum pedungulatum At 366 nm*

(ii). UV-Visible spectroscopy -

UV-Vis absorption in the range of 200-800 nm of the crude extract was observed using petroleum ether as the solvent. The spectrum shows maximum absorption at different wavelengths between the range of 200-800 nm. This helps us to characterize the double bonds.

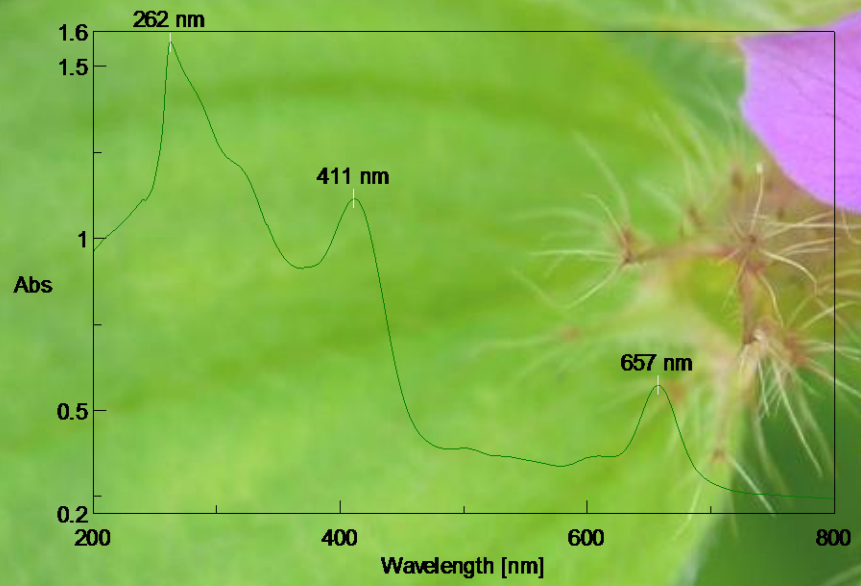


Fig 3: The UV-Vis spectrum shows three peaks of maximum absorption at 262nm, 411nm and 657 nm of petroleum extract due to $\sigma \rightarrow \pi^*$ transition which is characteristic of conjugated double bonds.

(iii). Infrared spectroscopy -

The sample was analyzed by FT-IR to identify the functional group.

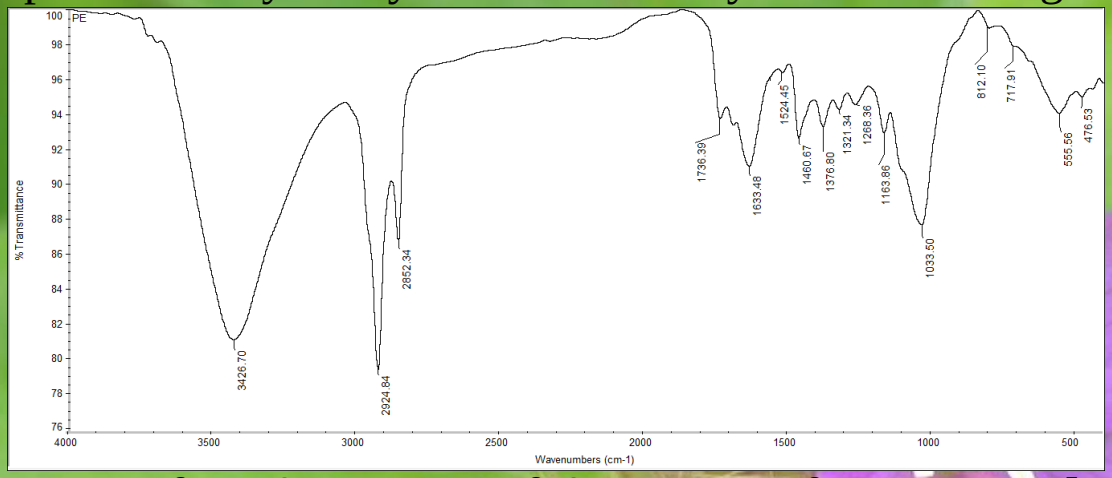


Fig 3 : Infrared spectrum of the extract of *Exacum pedunculatum*

Wavenumber (cm^{-1})	Band shape	Bond	Functional group
3426.70	Strong, broad	O-H, free hydroxyl	Stretching of phenols & alcohols
2924.84	Medium	C-H stretch	Alkanes
1736.39	Strong	C=O	Carbonyls
1633.48	Strong	C-H	Carboxylic acids
1524.45	Strong	N-O asymmetric stretch	Nitro compounds
1321.34	Strong	C-N stretch	Aliphatic amines

Table 3: Functional groups of the IR-spectrum of the petroleum ether extract.

(iv). Gas Chromatography-Mass Spectroscopy (GC-MS) -

The constituted extract was subjected to GC-MS analysis. The mass spectra of the compounds are identified based on the molecular mass, molecular structure and calculated fragmentations using the standard mass spectral database of WILEY and NIST.

The compounds identified by GC-MS analysis are –

1. N-hexadecanoic acid – anti-inflammatory and antihyperglycemic activity, anti- oxidant, hypocholesterolemic.
2. 1,13- tetradecadiene – Anti-inflammatory, component of essential oils.
3. 10-undecyn-1-ol – Anti-microbial, antifungal.
4. Hexanoic acid – Reduces blood cholesterol.

Table 4: Compounds identified by GC-MS

Compound Name	Molecular Wieght	Formula	Library
2,4,6,8-Tetramethyl-1-undecene	210	C15H30	NIST LIB
1-Octanol, 3,7-dimethyl-	158	C10H220	NIST LIB
2,4,6-Trimethyl-1-nonene	168	C12H24	NIST LIB
1-Dodecene, alpha-dodecene, n-dodecene	168	C12H24	NIST LIB
1-Hexanol, 3-methyl, 3-Methyl-1-Hexanol	116	C7H160	NIST LIB
1-Undecene, n-1-Undecene	154	C11H22	NIST LIB
1-Pentanol, 3,4-dimethyl-1-Pentanol	116	C7H160	NIST LIB
1-Heptanol	116	C7H160	NIST LIB
1-Nonene	126	C9H18	NIST LIB
Nonadecane	268	C19H40	NIST LIB
n-Hexadecane	226	C16H34	NIST LIB
2-methyloctadecane	268	C19H40	NIST LIB
Dodecane, 2,6,10-trimethyl-famesan	212	C15H32	NIST LIB
2,3,5,8-tetramethyl decane	198	C14H30	NIST LIB
n-Octasane	394	C28H58	NIST LIB
Pentadecanoic acid	242	C15H3002	NIST LIB
Tetradecanoic acid, myristic acid	228	C14H2802	NIST LIB
9,9-Dimethoxybicyclo(3,3,1) nona-2,4-dione	212	C11H1604	NIST LIB
Eicosanoic acid, arachidic acid	312	C20H4002	NIST LIB
Dodecanoic acid	200	C12H2402	NIST LIB
n-Hexadecanoic acid	256	C16H3202	NIST LIB
Octadecanoic acid, Stearic acid	284	C16H3602	NIST LIB
Heptadecanoic acid, heptadecyl ester	508	C34H6802	NIST LIB
Hexadecane	594	C40H8202	NIST LIB
2-Hexyl-1-decanol	242	C16H340	NIST LIB
n-Undecanal	170	C11H220	NIST LIB
n-Hexacosanol	382	C26H540	NIST LIB
Oleic acid, 9-Octadecenoic acid	282	C18H3402	NIST LIB
9-Hexadecenoic acid	254	C16H3002	NIST LIB
Isopropyl Myristate	270	C17H3402	NIST LIB

Computer-Aided Drug Designing

Outline of Docking procedure

- Open the website http://www.dockingserver.com/web/docking/
- Click on my protein.
- Click on download from PDB.
- Enter the protein code (1fm91) or the protein name(PPARγ).
- Enter the simulation box/grid box and click on confirm.
- Click on Ligand.
- Download from the Pubchem or draw the ligand.
- Click on Docking.
- Select the prepared protein and ligand from the list.
- Click on start dock.
- Automatically result with chart is displayed.

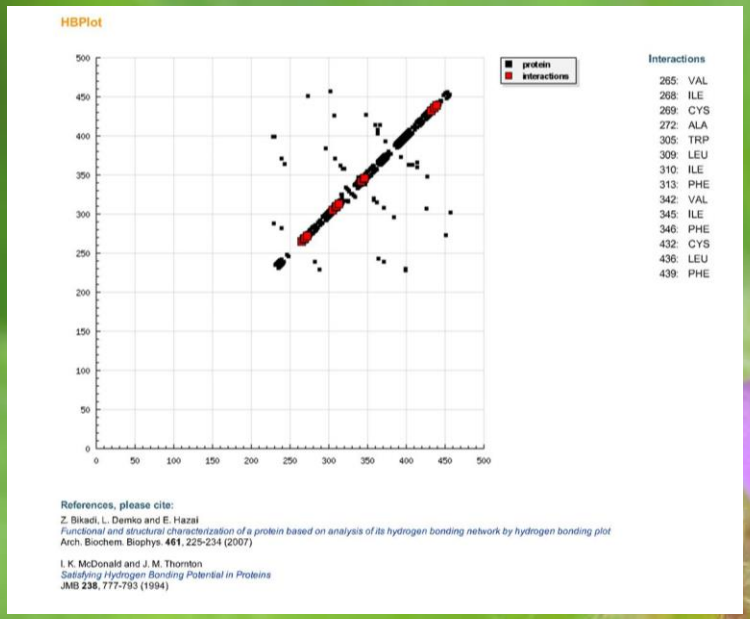
Computer-Aided Drug Designing

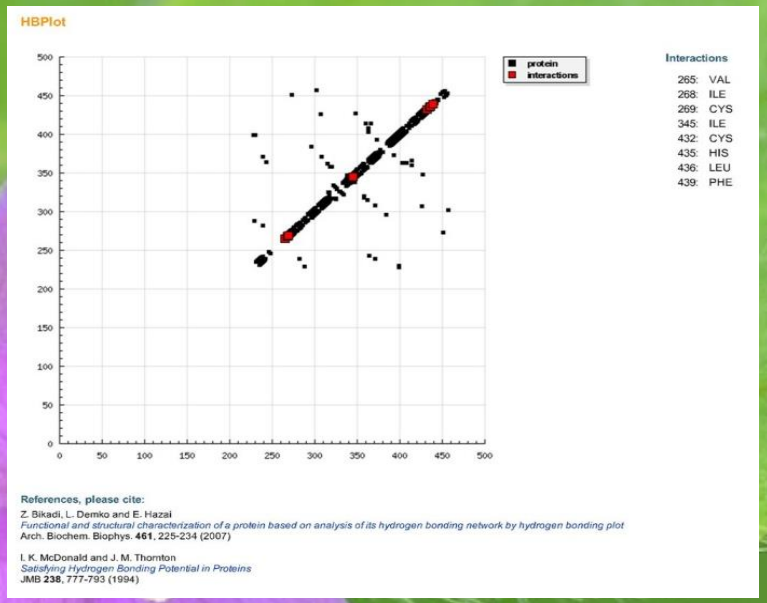
Physical Parameters

	Hexadecanoic acid	Metformin
Estimated Free Energy of Binding	**-5.57 Kcal/mol**	**-2.69 Kcal/mol**
Estimated Inhibition Constant ki	82.26mM	10.65mM
VdW+H bond+desolving Energy	-9.25 Kcal/mol	-2.59 Kcal/mol
Electrostatic Energy	0.00 Kcal/mol	-0.10 Kcal/mol
Total Intermolecular Energy	-9.25 Kcal/mol	-2.69 Kcal/mol
Frequency	50%	100%
Interaction Surface	546.75	321.773

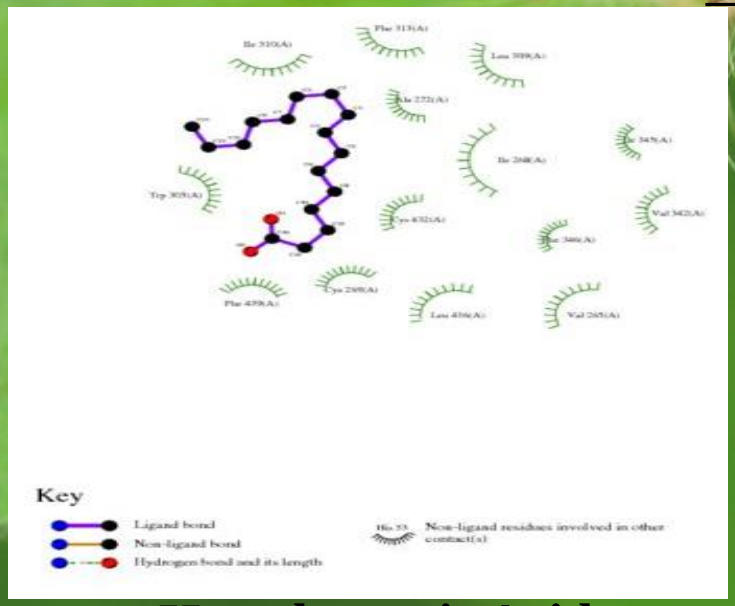
Parameters	Hexadecanoic acid	Metformin
Molecular weight	255.416	130.172
Molpol	30.71	13.00
logP	5.61	-0.30
pKa	4.95	-
pKb	0	11.60
Psa	37.30	91.49
logD	3.57	-3.07
Total charge	0.001	1.651
Log K	-13.556	0.918

Protein-Ligand Interaction

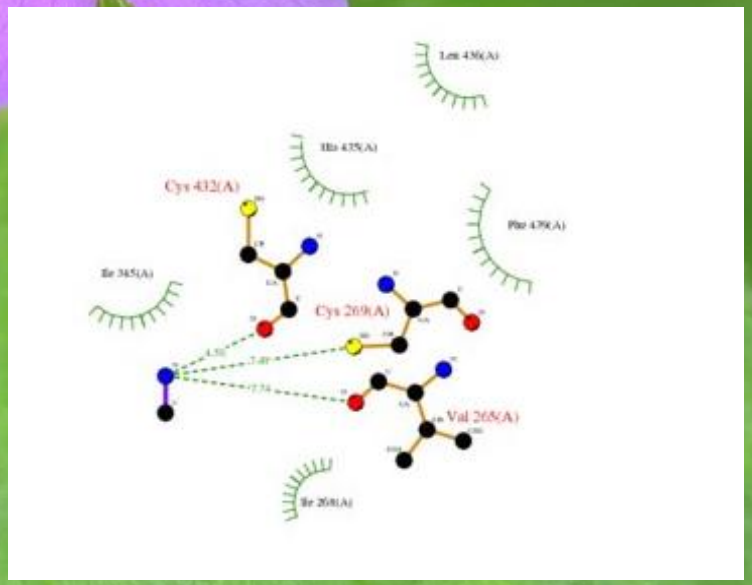




2 D Plot



Hexadecanoic Acid



Metformin

Contd...

Computer aided Drug Designing

Protien-Ligand interaction Ribbon Model

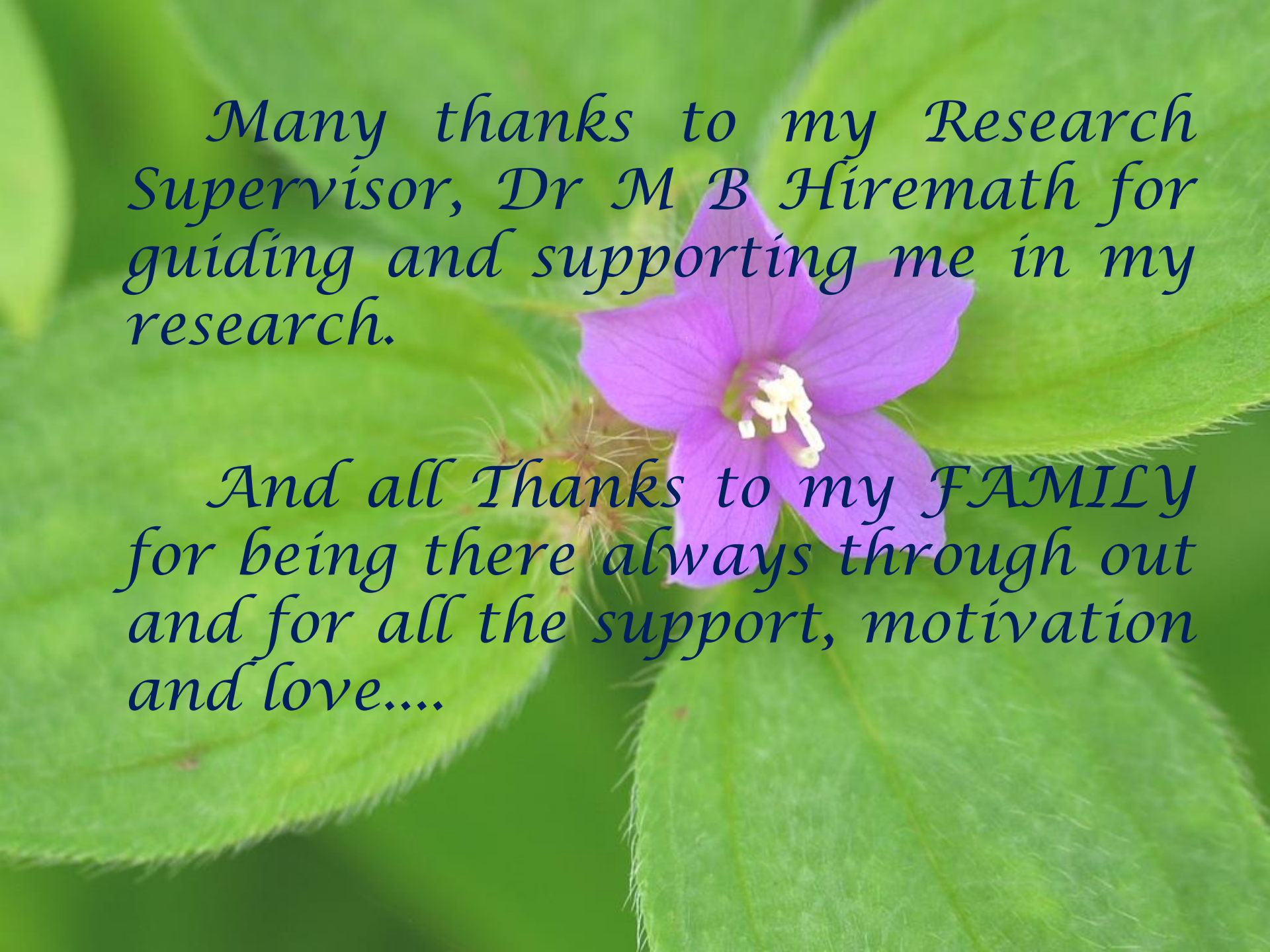
Discussion of the work done including findings.

Exacum pedunculatum, known as dodda chirayuta in Ayurveda is a rich plant containing various bioactive components.

The plant has been evaluated for the presence of major bioactive compounds and the structural analysis of the compounds would further help in establishing the mechanism of action of the compounds in *in vivo* studies.

Future Line of Work –

- *In vivo* testing of the identified compounds of the plant *Exacum pedunculatum* on animal model.


Many thanks to my Research Supervisor, Dr M B Hiremath for guiding and supporting me in my research.
And all Thanks to my FAMILY for being there always through out and for all the support, motivation and love....


"Nature has provided us generously with everything we need to remain in good health"
Thank You