

Research article

Identification of novel potent pancreatic lipase inhibitors from *Ficus racemosa*A. Vijaya Anand¹, V. Bharathi², G. Bupesh³, N. Jaya Lakshmi⁶, K. Meenakshi Sundaram³, K. M. Saradhadevi⁵¹Department of Human Genetics and Molecular Biology, ⁵Department of Biochemistry, Bharathiar University, Coimbatore, Tamil Nadu, India²Biological and Bioinformatics Research Centre, Trichy, Tamil Nadu, India³Department of Forest Science, Nagaland University, Lumami-798627, India⁴Research and Publication, Bharath University, Bharath Institute of Higher Education and Research, Chennai, Tamil Nadu, India⁶Department of Biochemistry, Shrimati Indira Gandhi College, Trichy, Tamil Nadu, India

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ABSTRACT

Introduction and Aim: Obesity is a disorder of lipid metabolism and continues to be a global problem, ranking fifth for deaths worldwide. It is considered to be main reason for a number of physiological changes that resulting in many metabolic disorders such as cardiovascular diseases, diabetes, musculoskeletal disorders and various cancer types. Obesity develops from long-termed physiological imbalances in energy expenditure.. One of the therapeutic strategies in managing obesity is inhibition of pancreatic lipase, a key enzyme responsible for the digestion of fats and triglycerides. The aim of the present study was to synthesize silver nanoparticles (Ag-NP) from *Ficus racemosa* fruit extract and to analyze its activity on pancreatic lipase enzyme using spectroscopic, *in vitro* and *in silico* methods.

Materials and Methods: The phytoconstituent were separated in *Ficus racemosa* fruit extract by thin layer chromatography technique. The bioactive compound was also identified by gas chromatography-mass spectrometry analysis. Pancreatic lipase inhibition assay was performed *in vitro* and confirmed by molecular docking analysis.

Results: The *F. racemosa* fruit showed the highest pancreatic lipase inhibitory activity. Molecular docking studies also exhibited good binding affinity of compounds (Diethyl Phthalate) with pancreatic lipase enzymes.

Conclusion: Finally, it is concluded that further derivation of such compounds could serve as the new templates for obesity.

Keywords: Obesity; pancreatic lipase enzymes; phytochemical; Protein Data Bank

INTRODUCTION

Obesity is a term related to the excess body weight with as an abnormally high proportion of the body fat that impairs health (1). Thermodynamically, disequilibrium between the energy intake and energy expenditure directs to the obesity (2). The obesity is generally caused by the combination of excessive food energy intake and lack of physical activity, Genetic factors, medical reasons, or psychiatric illness is the secondary causes. The increased rate of food consumption in the form of carbohydrates rather than fat consumption is an important cause of obesity (3). Orlistat (Xenical) is a therapeutic candidate for blocking the dietary fat absorption and seemed to be safe for long term usage. However, it causes unpleasant side effects (greasy stool), and you also need to supplement your diet with fat-soluble vitamins (4).

The deeper understandings of the process of lipid homeostasis, i.e., absorption, metabolism, transfer, storage, deposition and oxidation, have presented a wide variety of enzymatic targets involved (5). Dietary fats are mainly regarded as mixed triglycerides, which undergo a complex series of

biochemical reactions before absorption in the gastrointestinal tract (6). The medicinal plants constitute one of the important raw materials for drugs for treating various ailments of human being, although there has been significant development in the field of synthetic drug chemistry and antibiotics(7). Since, last two decades, the research world have witnessed a remarkable increase in scientific validation of the medicinal plants and their related compounds.. *Ficus racemosa* Linn. (Moraceae) is an evergreen, moderate to large sized spreading, lactiferous, deciduous tree 15-18 m high, without much prominent aerial roots (8). It belongs to the genus *Ficus* which is an exceptionally large pantropical genus with over 700 species and belongs to the family Moraceae which is distributed widely throughout the warmer parts of Asia, Africa, America and Australia. Experimental studies have demonstrated its anti-inflammatory, hepatoprotective and hypoglycemic effects (9). However, the reports related to the antihyperlipidemic effect of *F. racemosa* fruit are scarcely found elsewhere. In view of the above, it seems necessary to investigate *In vitro* lipase inhibitory activities of ethanolic extract of *F. racemosa* (10).

MATERIALS AND METHODS

Collection and authentication of plant materials

The fresh fruits of *Ficus racemosa* were collected from in and around areas of Manakkal village, Lalgudi, Tiruchirappalli, Tamil Nadu.

Preparation of seed and fruit powder

The fruits of *F. racemosa* were washed with sterile distilled water thrice, cut into small pieces and shade dried at room temperature for two weeks and made into a coarse powder using mechanical blender and stored in an airtight container.

Extract Preparation

F. racemosa fruit were first dried at room temperature and then grinded into fine powder by using electric grinder. Two different solvent extracts of *F. racemosa* fruits were prepared to screen the anti-lipase activity. Finely grinded *F. racemosa* fruit powder was subjected to aqueous and Ethanol, separately with ratio 10:100 in a flask and then kept it on shaker at room temperature on continuous shaking for 24-48 h. It was centrifuged for 15 min at 2000 rpm and supernatant layer was transferred to a new tube, then filtered through Whatmann filter paper 1. The filtrate then dried in a rotary evaporator at 50-60°C until all the solvents gets evaporated and only dry extract left behind. The obtained extracts were kept at 4°C condition until the further usage.

Phytochemical studies

The extracts of *F. racemosa* were screened for phytochemical constituents using standard procedures (11).

UV-VIS and FTIR Spectroscopic analysis

UV-visible spectrophotometric analysis was conducted on the *F. racemosa* extract using a UV-visible spectrophotometer (Perkin Elmer, USA Model: Lambda 950) with a slit width of 2nm, using a 10-mm cell at room temperature. The extract was examined under visible and UV light in the wavelength ranging from 300-800nm for proximate analysis. For UV-VIS spectrophotometer analysis, the extract was centrifuged at 3000 rpm for 10 min and filtered through Whatman No. 1 filter paper. The sample is diluted to 1:10 with the same solvent (12).

Fourier transform infrared (FTIR) was used to identify the characteristic functional groups in the extract (13). A small quantity of the *F. racemosa* fruit extract was mixed in dry potassium bromide (KBr). The IR spectrum was obtained using Bruker, Germany Vertex 70 infrared spectrometer. The sample was scanned from 4000 to 400 cm⁻¹ (13). The peak values of the UV-VIS and FTIR were recorded.

Thin-layer chromatographic study [TLC]

For TLC studies, the TLC plates were prepared by using the Silica gel 'G'. About 30 gm of silica gel was made to a homogenous suspension with 60 ml distilled water and distributed over the plate. After air dried, the plates were kept in a hot air oven at 110°C for 30 min and stored. Samples were prepared by diluting the extracts and applied over TLC plate 2cm above its bottom and kept in the chromatographic chamber. The tests were performed for alkaloids, flavonoids, tannins and phenols, solvent systems during the movement of the mobile phase on its ¾ distance..

Compound identification and separation

Gas Chromatography-Mass Spectrometry analysis

30 g powdered sample of *F. racemosa* were soaked and dissolved in 75 ml of ethanol for 24 hours and the filtrate was evaporated on liquid nitrogen environment. The Gas chromatography-mass spectrometry analysis was carried out using a Clarus 500 Perkin-Elmer (Auto System XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mass Gold-Perkin Elmer Turbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl poly siloxane), 300 m X 0.25 mm Xx 1 µm df capillary column. The initial temperature was kept as 110°C then, the instrument was maintained for 2 minute at the same temperature. Then, the oven temperature was adjusted to 280°C by increasing 5°C/min evenly. This temperature was maintained for for 9 min. The injection port temperature was ensured as 250°C and helium flow rate as 1 ml/ min. The ionization voltage was 70 eV. The samples were injected in split mode (10:1) and the spectra range was kept as 45-450 (mhz). The obtained resultant spectral fragmentation patterns was compared with the library of National Institute of Standards and Technology Mass Spectral database (NIST-MS). The quantity of the individual compounds (%) was calculated by their relative peak area as present in the chromatogram.

Pancreatic lipase inhibition assay

The pancreatic lipase activity was quantified by a colorimetric assay that measures the release of *p*-nitrophenol as previously described (14) with minor modification. The enzyme solutions were prepared immediately before use. Crude porcine pancreatic lipase type II (Sigma, EC 3.1.1.3) was suspended in Tris-HCl buffer (2.5 mmol, pH 7.4 with 2.5 mmol NaCl) to give a concentration of 5 mg/mL (200 units/mL) and mixed using a stirrer for 15 min. It was centrifuged at 1500g for 10 min and the supernatant was incubated with the test solutions. The 0.10 mL of supernatant solution was preincubated with different concentrations (1.56-100 µg/mL) of the extract and the selected compounds for 5 min at 37°C, and then the PNPB substrate (10 mM in

acetonitrile) was added. It was further diluted to 1 mL using the Tris-HCl buffer. Its ratio was measured spectrophotometrically at 410 nm at least five time points: 1-5 min. The release of *p*-nitrophenol is measured as the increase in absorbance at 410 nm against blank using denatured enzyme. DMSO was used as negative control and its concentration was not exceeded higher than 2.0%. The slope of the linear segment of absorbance vs. time profiles based on the release of *p*-nitrophenol. The pancreatic lipase activity could be calculated by the rate of *p*-nitrophenol release. The percentage of residual activity of pancreatic lipase was determined for each compound by comparing the lipase activity of pancreatic lipase with and without the compound. All assays were triplicated; thus, inhibition percentages are the mean of triplicate observations. Orlistat, a known inhibitor of pancreatic lipase, was used as a positive control in the assay mixture

In vitro Molecular docking analysis

The crystallographic structure of ATP-binding pocket of pancreatic lipase was downloaded from Protein Data Bank (PDB ID: 3L2M). The canine smiles of diethyl phthalate were downloaded from PubChem data base and its 3D structure was generated by Chemdraw ultra (ver.10.0) software. Docking studies were performed to reveal the binding pattern of the individual compounds with the target proteins. The

molecular docking analysis was done Autodock software (version 4.2.6). The identified potential compounds after GC-MS analysis were used as ligands for the study. The possible interactions of the best-scored solution of the models for each ligand were analyzed (15).

Statistical analysis

The experimental calculations were performed using Microsoft 2010 and GraphPad Prism 6.0 software. The mean and standard deviation was significance $P < 0.05$.

RESULTS

Phytochemical analysis

The preliminary phytochemical analysis was carried out in the extracts of *F. racemosa*. The phytochemical analysis was carried out in the two different extracts (Table 1). The ethanolic extract of *Ficus racemosa* showed on indication of the presence of saponin, coumarin, flavonoids, tannin, phenolic compound, and quinone were confirmed in the suitable chemical test. The aqueous extracts of *F. racemosa* contain alkaloids, tannins, saponins and phenolic compounds. Moreover, the highest yield was also observed in ethanolic extract. So, the ethanolic extract was used for present study.

Table 1: Phytochemical screening of *F. racemosa* extracts

S. No.	Phytochemical	Water	Alcohol
1	Alkaloids	+	+
2	Flavanoids	+	+
3	Steroids	+	+
4	Tannins	+	+
5	Terpenoids	-	+
6	Quinine	+	+
7	Coumarins	-	+
8	Volatile oil	-	-
9	Saponins	+	+
10	Phenols	+	+

Thin layer chromatography analysis

Thin layer chromatography analysis also suggests the presence of different kinds of phytochemicals in fruit extract. Thin layer chromatography was performed on plant extracts using different solvent systems ethanol: water: chloroform (5:1:4). Thin layer

chromatography of plant extracts in chloroform reports three spots for various phytochemicals. The reported spots are separated with enough space and having various R_f values showing the presence of at least three phytochemicals in ethanol extracts (Table 2 and Fig. 1).

Table 2: Thin layer chromatography of *F. racemosa* extracts

S. No.	Extracts	Spot	R_f Value
1	Crude water (a)	Brown Spot	0.50
2	Crude alcohol (b)	Green Spot	0.83



Fig. 1: Thin layer chromatogram of *F. racemosa* extracts

UV-VIS analysis

The qualitative UV-VIS profile of ethanolic extract of *F. racemosa* was taken at the wavelength of 300 nm to 800nm due to the sharpness of the peaks and proper baseline. The profile showed the peaks at 278

and 457nm with the absorption 4.000, and 1.5088 respectively. Fig. 3 shows the absorption spectrum of *F. racemosa* extract and these are almost transparent in the wavelength region of 300-800 nm (Fig. 3 and Table 2).

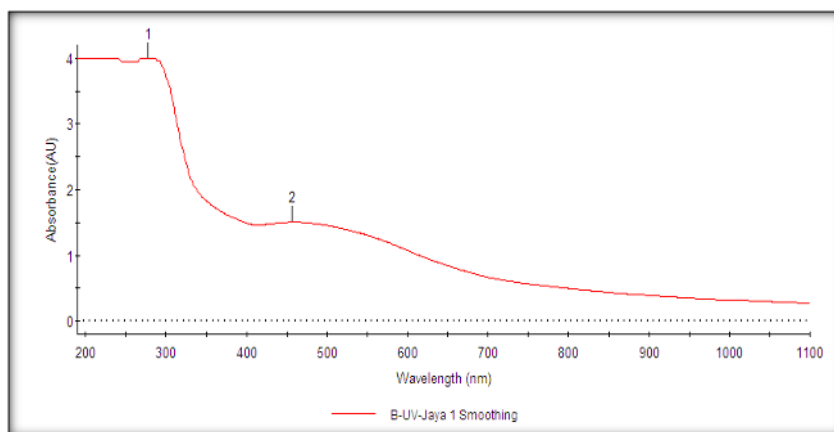


Fig. 2: UV-VIS analysis of *F. racemosa*

Table 3: UV-VIS Spectrum analysis of *F. racemosa*

S. No.	Wavelength	Absorbance
1	278.00	4.0000
2	457.10	1.5088

FT-IR analysis

FT-IR measurement was carried out to identify the possible biomolecules responsible for antiobesity activity using *F. racemosa* extract. This spectrum shows lots of absorption bands (Fig. 3) indicates the presence of active functional groups in the *F. racemosa*. The intensity peaks are slightly increased

for the period of 3369,2130,1642,3444,1287 cm^{-1} as well as some intensity peaks decreased like 1081,1045 and 600 cm^{-1} . Figure 4 shows the band at 3369 correspond to 1°,2° amines, amides. The peak at 2130 represents to C-H in plane bend to alkenes.

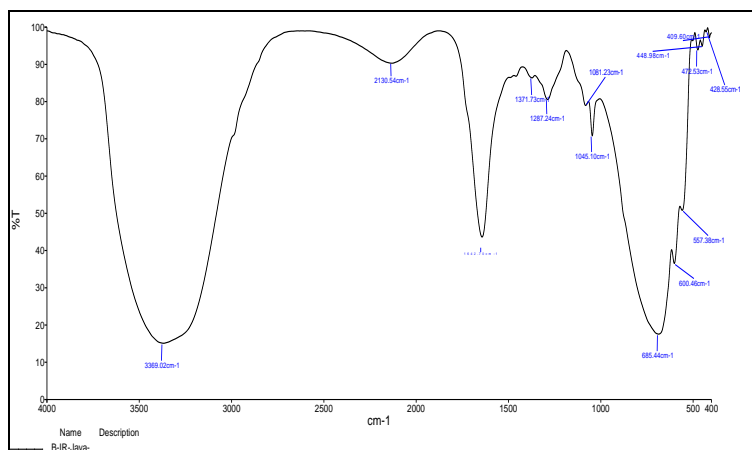


Fig. 3: FT-IR analysis of *F. racemosa*

GCMS analysis

The compounds present in the ethanolic extract of *Ficus racemosa* were identified by Gas chromatography-mass spectrometry analysis (Table 4 and Fig. 4). The active principles with their retention

time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in Table 4 and Fig. 4. The GC MS analysis showed that the extract was rich in phyto-constituents.

Table 4: GCMS analysis of *F. racemose*

Peak	Area	Height	Name
1	283075	239699	Oxime-, methoxy-phenyl-_
2	831401	444929	Benzoic Acid, 2-Hydroxy-, Methyl Ester
3	688563	400467	Benzoic acid, 2-hydroxy-, ethyl ester
4	30721868	11480528	Diethyl Phthalate
5	186730	81073	1-(+)-Ascorbic acid 2,6-dihexadecanoate
6	16505	11834	Nonanoic Acid, Ethyl Ester
7	28684	9553	Bicyclo(4.1.0)heptane, 3-methyl-
8	299231	53145	Oleyl alcohol, trifluoroacetate
9	88258	22322	2-Aminoethanethiol Hydrogen Sulfate (Ester)
10	32110	14377	2-Methylcyclopentanon-Oxime
11	125487	36296	Spirost-5-en-3-ol, (3.beta.,25r)-
12	259247	54349	Spirost-5-en-3-ol, (3.beta.,25r)-

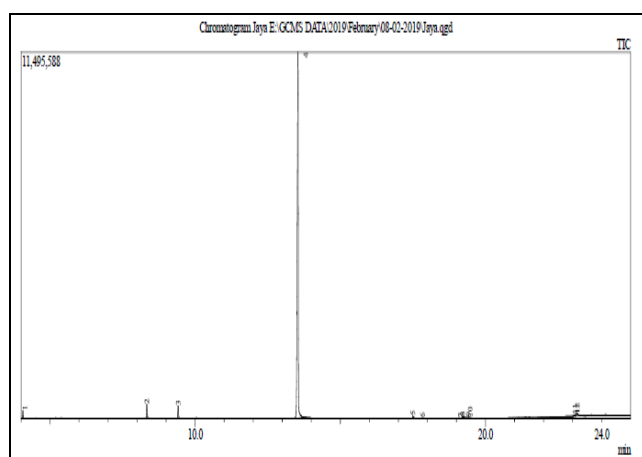


Fig. 4: Gas Chromatography-Mass Spectrometry analysis

In vitro pancreatic lipase inhibitory activity of *F. racemosa*

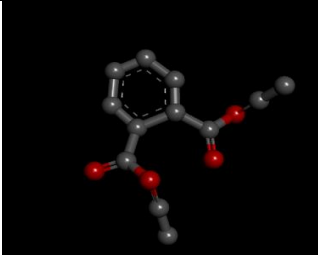
The dried fruits of *Ficus racemosa* were successively extracted with water. The resulting crude extracts were evaluated for anti-lipase activity using p-nitrophenylpalmitate as substrate. The pancreatic

lipase activity of *Ficus racemosa* extract is depicted in Table 5. The *Ficus racemosa* showed dose-dependent pancreatic lipase inhibitory activity. The fruits of *Ficus racemosa* showed 46.2%, 44.4%, 39.33%, 21.89 and 12% inhibition, respectively. The maximum inhibitory activity was observed in concentration of 25 µG/ML.

Table 5: *In vitro* pancreatic lipase inhibitory activity of *F. racemosa*

S. No.	Concentration of sample ($\mu\text{G/ML}$)	% of inhibition of <i>F. racemosa</i>	% of inhibition of Orlistat
1	05	12.00	15.0
2	10	21.89	30.0
3	15	39.33	45.0
4	20	44.4	60.0
5	25	46.12	75.0

Table 6: Structure of a phytocompound from *F. racemosa*

S.No.	Compound name	Canonical smiles	Compound structure
1	Diethyl Phthalate	<chem>O=C(OCC)C1=CC=CC=C1C(OCC)=O</chem>	

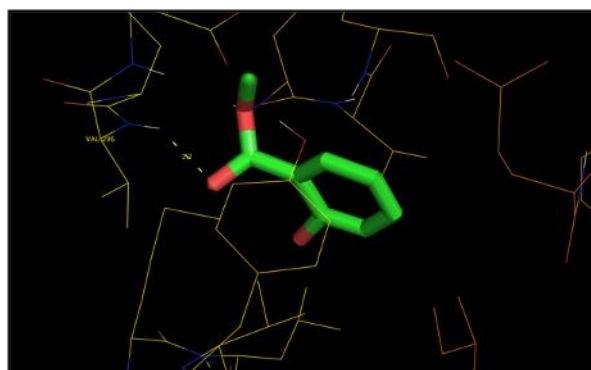
Docking studies

Docking was carried out by AutoDock4 in ATP-binding pocket. Grid map in Autodock that defines the interaction of protein and ligand in binding pocket was defined. The grid map was used with 60 points in each x, y, and z direction, equally spaced at 0.375\AA . Docking was performed using the Lamarckian genetic algorithm. Each docking

experiment was performed 200 times, yielding 200 docked conformations. The parameters used for the docking were as follows: population size of 150; random starting position and conformation. The results of the molecular docking analysis indicate that the compound from *F. racemosa* were more selective towards the ATP-binding pocket (Fig. 5) of pancreatic lipase (PDB ID: 3L2M).

Table 7: Molecular docking analysis of compound from *F. racemosa* with binding pocket of pancreatic lipase (PDB ID: 3L2M)

S. No.	Compounds	Binding energy	H-Bond interaction	H-Bond distance \AA
1	Diethyl Phthalate	-5.96	VAL 296...O1	2.2

**Fig. 5:** Docking pose of compound 1 (diethyl phthalate) on binding pocket of pancreatic lipase (PDB ID: 3L2M) (Yellow line represents hydrogen bond interactions)

DISCUSSION

Plant Polyphenolics commonly indicate the group of phytocompounds including phenolic acids, flavonoids, natural dye, lignins etc. Usually, those are produced by plants as a secondary metabolite which represent a potential source with a significant amount of antioxidants to prevent oxidative stress caused by

free radicals (16). The methanolic extract of *Indigofera trita* was reported to possess polyphenolic compounds exhibits its antioxidant activity by chelating redox-active metal ions, in activating lipid free radical chains and preventing hydroperoxide conversion into reactive oxyradicals and other biological properties includes diffusion of toxic free

radicals, altering signal transduction, activation of transcription factors and genes expression by previous study (17).

In our study, ethanol: water: chloroform (5:1:4) was used to separate the phytochemicals since, it showed the largest discriminating power. Three bands found in this method and its R_F values were 0.50 and 0.83. This value indicates the presence of phenolic compound and concurred with the previous study (18). UV- VIS analysis revealed the presence of compounds that characteristically showed sharp bands. These are characteristics bands of flavonoids and their related derivatives. Usually, the flavonoid spectra consist of two absorption maxima between 230-285 nm (band I) and 300-350 nm (band II). The nature of the flavonoids could be derived from the precise position of the individual bands and their relative intensities this is in accordance with the previous literature on *Acorus calamus* (19).

Further, in our study, FTIR analysis was carried out to show the prominent functional groups present in the active compounds. It showed that the peak at 1287 corresponds to C-N stretch vibrations to aromatic amines. The weak band at 600 indicates C-Br stretch stretching vibrations, it corresponds to the presence of Alkyl halides, and aliphatic amines in the plant extract. The functional group the individual compounds could be derived from the FT-IR spectrum. Similarly, *Cayratia trifolia* plant stem's ethanolic extract holds more phytochemical and bioactive compounds, which were confirmed using FT-IR (20).

The aqueous extract of *F. racemosa* had prominent pancreatic lipase inhibitory potential in the study as reported previously (23). Gastric and lingual lipases are responsible for partial hydrolysis of dietary into free fatty acids and diacylglycerols. This partial digestion in stomach forms large fat molecule which undergoes emulsification with bile salts to form small droplets of fat. A physical property of emulsion influences the efficiency of the digestion. In the emulsion, dietary triglycerides and diglycerides in the center of droplet followed by a mixture of polar lipids, phospholipids, cholesterol, and free fatty acids and later coated with oligosaccharides, denatured proteins and bile salts and thus forming a complex structure. The pancreatic lipase interacts with this complex structure, which continuously changes its physical properties as products formed, leaves the surface during the process of hydrolysis. Complete hydrolysis process results into free fatty acids, monoacylglycerols, and diacylglycerols bind with cholesterol, bile salts, fat soluble vitamins and lysophosphatidic acid to form mixed micelles which can be absorbed by enterocytes. Pancreatic lipase uses a pancreatic protein colipase, as cofactor, to facilitate lipolytic activity. The aqueous *Opuntia ficus-indica* extract is able to prevent

hypercholesterolemia by pancreatic lipase inhibition, in part due to its polyphenolic compounds. (24).

The docked poses were categorized using their Lowest Binding Energy (LBE), Hydrogen bond interactions. The expected binding energy was found between -5.96 kcal/mol. These binding energy values indicate that the newly synthesized compounds had shown a fortunate selectivity towards ATP-binding pocket of pancreatic lipase (PDB ID: 3L2M) The 2D view of protein-ligand interactions of the best poses generated by 1AJ6 studied routines are shown in Fig. 6. All the top docked poses generated by each docking routine exhibited well-established bonds with one or more amino acids in the binding pocket of receptor (5, 9, 12). Especially compound from *F. racemosa* showed hydrogen bonds with shorter distance revealed the strength of the interaction. From these results, our study showed that the synthesized compound, diethyl phthalate have the ability to inhibit the pancreatic lipase enzyme by accommodating itself in ATP-binding pocket which might be a reason for good activity against obesity. Our study warrants for further molecular level analysis to bring out the potential drug candidature of Ag synthesized diethyl phthalate on obesity.

CONCLUSION

The present study revealed the *F. racemosa* ethanolic extract contain more phytochemicals when compared to aqueous extract. The bioactive compounds such as Oxime-, methoxy-phenyl-Benzoic Acid, 2-Hydroxy-, Methyl Ester Benzoic acid, 2-hydroxy-, ethyl ester Diethyl Phthalate 1-(+)-Ascorbic acid 2,6-dihexadecanoate Nonanoic Acid, Ethyl Ester Bicyclo(4.1.0)heptane, 3-methyl- Oleyl alcohol, trifluoroacetate 2-Aminoethanethiol Hydrogen Sulfate (Ester) 2-Methylcyclopentanone-Oxime Spirost-5-en-3-ol, (3.beta.,25r)- Spirost-5-en-3-ol, (3.beta.,25r)- were identified by Gas chromatography-mass spectrometry analysis. The fruit extract showed the highest pancreatic lipase inhibitory activity. Molecular docking studies also exhibited good binding affinity of compounds (Diethyl Phthalate) with pancreatic lipase enzymes. Finally, it is concluded that further derivation of such compounds could serve as a new drug for obesity.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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