PHYTOCHEMICAL ANALYSIS, EVALUATION OF BIOACTIVE COMPOUNDS IN *Piper nigrum* Linn AND INTERACTION STUDIES OF PIPERINE WITH *Aeromonas hydrophila* (aerA)

[1]A Zahira, [2]K Thamilmani, [3]R Rafi Mohamed, [4]M Mohammed Sahinsha
[1&4]Research Scholar, [2&3]Assistant Professor,
[1&2] PG & Research Department of Zoology, Arignar Anna Government Arts College, Musiri – 621 211, India
[3]PG & Research Department of Zoology, C Abdul Hakeem College, Melvisharam -632 509, India
[4]Department of Pharmaceutical Technology, Anna University, Tiruchirappalli- 620 024, India

Abstract : The bioactive phytocomponents of *Piper nigrum* Linn. seeds have been analyzed and evaluated using GC-MS, RP-HPLC and FT-IR techniques. The chemical compositions of the extract of *P nigrum* seeds were investigated using Clarus 500 Perkin-Elmer Gas Chromatography–Mass Spectrometry. The mass spectrum of the compounds was matched by the National Institute of Standards and Technology (NIST) library database. GC/MS analysis of extract of *P nigrum* seeds revealed the existence of Piperine (26.6745 %), o-Anisic acid, 2-adamantyl ester (17.9390%), Eugenol (9.5204%), Furane-2-carbohydrazide, 5-phenylethynyl- (6.5202%) and Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene, [1R-(1R*,4Z,9S*)]- (4.8802%). RP-HPLC profiles of *P nigrum* reported to contain 10.963% of the piperine in the extract based on peak area obtained. The results of FT-IR analysis confirmed the presence of amide, ether, aliphatic and aromatic alkene and alkane functional groups. Molecular docking analysis of the ligand piperine in the homology modeled protein of *Aeromonas hydrophila* aerA (Aerolysin) shows the good binding energy of -7.2 kcal/mol. This study forms a source for the biological characterization of phytocompounds identified and justifies the usage of *P nigrum* as an herbal alternative for treating various diseases.

Keywords: GC-MS, RP-HPLC, FTIR, *Piper nigrum*, Molecular Docking, Aerolysin, Aeromonas hydrophila, aerA

INTRODUCTION

Plants are very rich source of secondary metabolites with a variety of structural arrangements and interesting pharmacological activities[1]. Subcontinent of India is one of the richest countries in genetic diversity of medicinal plants. As per the statement of World Health Organisation, more than 80% of the world's population depends on the traditional medicine for their primary healthcare needs [2]. For centuries, people have been trying to alleviate and to treat various illnesses with different medicinal plant extracts and formulations[3]. Moreover, the medicinal plants are cheaper, safe, widely available and with no or lesser incidence of unwanted side effects. Natural products play an important role in the development of novel drug leads for the prevention and treatment of diseases[4-6]. Increasing awareness in medicinal products of natural origin has today accelerated the growth of medicinal plant- based industries[7].

The genus piper (Family: Piperaceae) comprised of more than 1000 species and they are widely distributed in the tropical regions of the world. *Piper nigrum* Linn. plant is a woody perennial climber indigenous to Southern[8] and South easetern Asia. It is commonly known as black pepper, pepper, etc., Due to its wide usage as condiment and spices worldwide, it is considered as "King of Spices" and "Black gold". Major producers of black pepper are Vietnam, Indonesia, India and Brazil[9]. *P nigrum* Linn. is perennial, branching, stout climber with glabrous stems and rooting at the nodes. Leaves are simple, alternate, cordate, ovate to elliptic, pointed at apex, 5 to 9 nerved and dark green colored. Flower spikes are usually deciduous.

Fruiting spikes are 7 to 10 cms long, robustness, glabrous rachies and arranged loosely, fruit drupe, globular, red coloured when ripe which turns to black color after drying. Being a pungent condiment, the fruit is used in many recipes worldwide as a common ingredient. Major constituents of pepper include an alkaloid piperine, volatile oil, pungent resin and piperidine. *P. nigrum* can be used for its Antihypertensive, Antiasthmatic, Fertility activity, Antimicrobial, Antioxidant, Anticancer, Anti-inflammatory, Hepato-protective, Anti-diarrhoeal, Digestive, Antidepressant, Immunomodulatory, Anticonvulsant and Analgesic activities [10-21].

The study of the pharmacologically active secondary metabolites present in the plant is very important because most drugs were synthesized after a careful study of phytoconstituents and their structures[22]. GC – MS is a hyphenated system which combines the features of gas-liquid chromatography and mass spectroscopy for the identification and quantification of different substances present in the test sample. Application of GC/MS technique includes the detection of drug, environmental analysis, investigation of explosives and recognition of unknown samples. GC-MS spectral analysis was also carried out to identify the name, molecular weight and structure of the components present in the test samples. The unknown organic compounds in the complex mixture can be determined by interpretation as well as by matching the spectra with reference spectra[23-24].

Aeromonas hydrophila is the most common bacterial pathogen in freshwater fishes and has been recognized as an aetiological agent of pathological conditions like tail rot, motile haemorrhagic septicemia and epizootic ulcerative syndrome as a primary pathogen [25]. The continuous usage of conventional antimicrobials in aquaculture has resulted in more resistant bacterial strains in the aquatic environment [26,27]. Because of the increasing bacterial resistance, the searches for new active substances from natural origin are of increasing importance [28]. Keeping these points in view, the present research has been carried out to investigate the phyto-constituents present in the seed extract of *P. nigrum* Linn. and its *in-silico* activity screening for their antimicrobial activity.

MATERIALS AND METHODS

Collection and Identification of *P nigrum* L.

Seeds of *P nigrum* L. were collected from Palakkad District, Kerala, South India. Plant material was identified by examination of the morphological characters, authenticated and voucher specimen (Specimen No.: QDK005) was deposited in Rapinet Herbarium, St. Joseph's College, Tiruchy, Tamil Nadu, India.

GC-MS Spectral Analysis [29] :

The powdered sample (20 g) were soaked and dissolved in 75 ml of methanol for 24 hrs. Then the filtrates were collected and evaporated under liquid nitrogen. The GC-MS 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 – Perking Elmer Turbomas 5.2 spectrometer with an Elite-1 (100% Dimethyl polysiloxane), 30 m x 0.25 mm x 1 x m df capillary column. The instrument was set to an initial temperature of 110°C for 2 min and the oven temperature was raised up to 280°C, at the rate of an increase of 5°C/min and maintained for 9 min. Injection port temperature was ensured as 250°C; Helium flow rate as 1.0 ml/min; and the ionization voltage as 70 eV. The samples were injected in split mode as 10:1. Mass Spectral scan range was set at 45-450 mhz. The chemical constituents were identified by GC/MS. The fragmentation patterns of mass spectra were compared with those stored in National Institute of Standards and Technology Mass Spectral (NIST-MS) database. The percentage of each component was calculated from relative peak area in the chromatogram.

RP-HPLC Purification and Quantification [30] :

The dry powders was extracted individually thrice with Methanol : Ethyl acetate with 1:1 ratio, the solvent fraction was eluted using solvent fractionator and resulting solvent was evaporated under vaccum (Lablinks PBU-6, India). This extract were re-dissolved in 5 ml of methanol, filtered through a 0.2 µm syringe filter and separated by Reverse Phase (RP)-HPLC. RP-HPLC was performed using a Waters 600 HPLC system (Waters, USA) equipped with an Xterra Prep RP18 OBD column (Waters, USA; 5 µl, 18

× 100 mm) held at 40°C. The solvent system consist of distilled water (solvent A) and acetonitrile (solvent B). The compounds were eluted at a flow rate of 4 ml/min with a linear gradient from the mixture A:B (100:0, vol/vol) to A:B (0:100, vol/vol) in 45 min. The absorbance of the eluate was measured at 210 nm. All the collected fractions were dried and stored at - 20°C.

Chemical characterization by FT-IR [31] :

10mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, in order to prepare translucent sample disc. The powdered sample of *P nigrum* was loaded in FTIR Spectroscope (Shimadzu, IR Affinity-1 FTIR, Japan), with a scan range from 400 cm^{-1} to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1}. Fourier-transform infrared spectrophotometer (FT-IR) is a powerful tool to identify the type of functional groups present in the tested compound. The peak values of the FTIR were recorded twice for the spectrum confirmation.

Homology Modelling: [32-36]

The amino acid sequence of *Aeromonas hydrophilla* was retrieved from NCBI (AEC12846.1). A three dimensional model was generated for "*Aeromonas hydrophilla*". A sequence similarity search was performed to identify the structural similarity of the query sequence by using Protein Blast tool by selecting database against Protein Data Bank (PDB) for identifying template for homology model building. The template was identified on the basis of searching the E-value, above 90% identity, maximum score. 1PRE (PDB CODE) protein was selected as a template for modeled protein.

Molecular Docking Studies: [37-39]

Piperine was docked by using Autodock 4.2 software. The modelled three dimensional structure of Aeromonas hydrophilla aerA protein was imported to Autodock 4.2 and structurally optimized by adding hydrogens to protein allocated with computer Gasteiger charges. After adding the hydrogens the model was saved in PDBQT format, later ligand was prepared by optimizing the torsion angles and saved them in PDBQT format. A grid was generated around to identify XYZ coordinates (X= 60, Y=60 and Z= 60), center value parameter of xyz was set to (X= -3.705, Y=47.77 and Z= 37.92) around binding site of *Aeromonas hydrophilla* aerA protein. Lamarckian Genetic Algorithm (LGA) was selected for freezing, docking and default parameters are used in autodock 4.2. The interaction between the protein and ligand was analyzed by Discovery Visualizer.

RESULTS AND DISCUSSION :

GC-MS ANALYSIS :

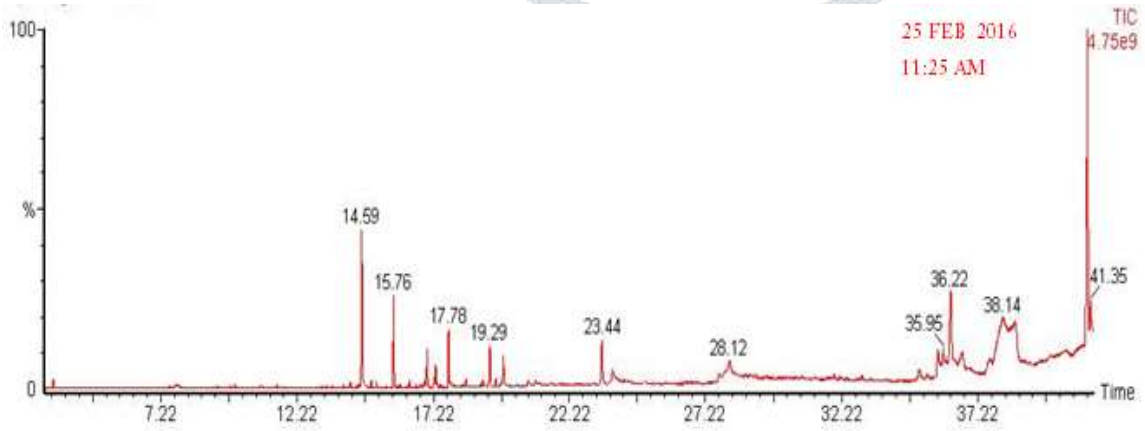


Fig. 1 : GC - MS spectra of methanolic seed extract of *P nigrum* L.

Phytochemical compounds present in the methanolic seed extract of *P. nigrum* were identified by GC-MS analysis. Totally 55 peaks were recorded; phytoconstituents were characterized, identified and tabulated in Table-1. The high peak compounds in this sample were Piperine **(**26.6745%), Bicyclo [7.2.0] undec-4-ene,

4,11,11- trimethyl -8- methylene-,[1R-(1R*,4Z,9S*)]- (4.8802%), Eugenol (9.5204%), o-Anisic acid, 2-adamantyl ester (17.9390%). The GC-MS chromatogram of *P. nigrum* is shown in Figure 1. All these compounds were pharmacologically important and they have the properties such as analgesic, antidiabetic, antibacterial and antifungal activity.

Table - 1: Qualitative and quantitative determination of biochemical constituents in *P nigrum* by GC-MS

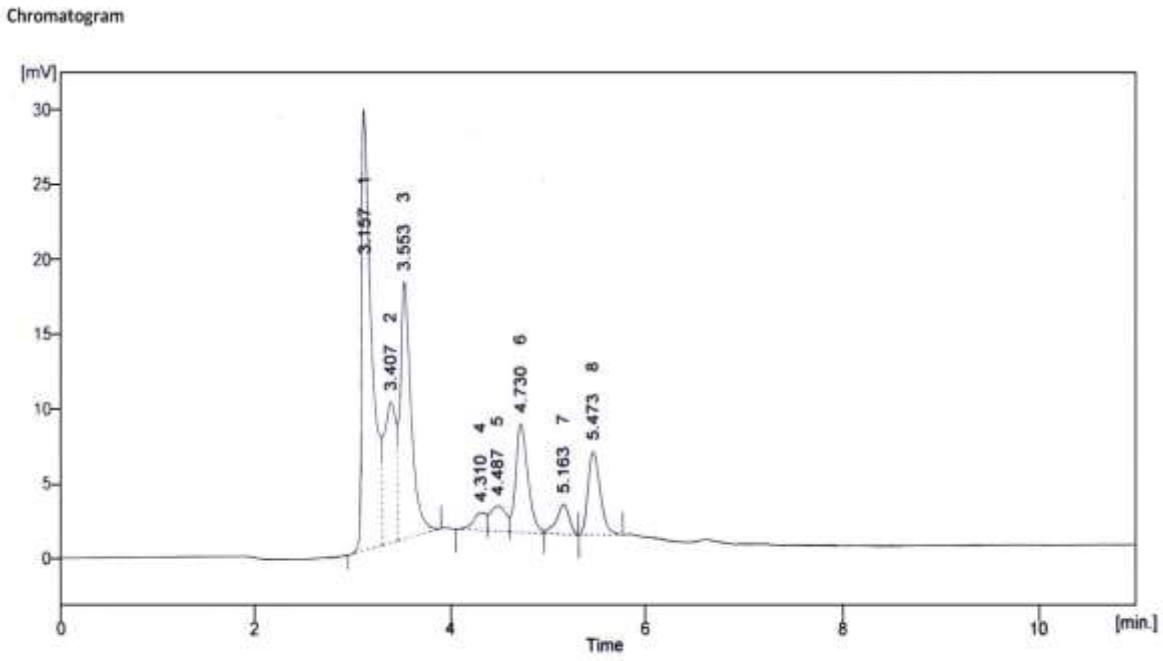
Peak No.	Peak name	Mol. Wgt	Chemical Formula	Retention time (min)	%Peak Area
1	Acetic acid, 1-methylethyl ester	102	$C_5H_{10}O_2$	3.28	0.3104
2.	Methylglyoxal	72	$C_3H_4O_2$	4.06	0.1177
3.	2,3-Butanediol, [S-(R*,R*)]-	90	$C_4H_{10}O_2$	4.21	0.0879
4.	à-Phellandrene	136	$C_{10}H_{16}$	6.38	0.0474
5.	2-Cyclopenten-1-one, 2-hydroxy-	98	$C_5H_6O_2$	6.53	0.0583
6.	Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	136	$C_{10}H_{16}$	7.32	0.0763
7.	1-Piperidineethanamine	128	$C_7H_{16}N_2$	8.42	0.0540
8.	Cyclohexene, 4-methyl-1-(1-methylethenyl)-	136	$C_{10}H_{16}$	8.98	0.0283
9.	Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1à,2à,5à)-	154	$C_{10}H_{18}O$	9.31	0.0939
10	dl-Malic disodium salt	134	$C_4H_6O_5$	9.41	0.0149
11	1,6-Octadien-3-ol, 3,7-dimethyl-	154	$C_{10}H_{18}O$	9.79	0.1109
12	Terpineol, cis-á-	154	$C_{10}H_{18}O$	9.94	0.1509
13	1-Piperidinecarboxaldehyde	113	$C_6H_{11}NO$	10.83	0.0623
14	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	154	$C_{10}H_{18}O$	11.48	0.1346
15	Name: 3-Cyclohexene-1-methanol, à,à4-trimethyl-	154	$C_{10}H_{18}O$	11.75	0.0610
16	4-Thujen-2à-yl acetate	194	$C_{12}H_{18}O_2$	11.95	0.0507
17	Limonene oxide, trans-	152	$C_{10}H_{16}O$	12.50	0.0740
18	3-Nonanol, 1,2;6,7-diepoxy-3,7-dimethyl-, acetate	242	$C_{13}H_{22}O_4$	13.12	0.1039
19	1-Piperidineethanamine	128	$C_7H_{16}N_2$	13.27	0.1507
20	1,3-Benzodioxole, 5-(2-propenyl)-	162	$C_{10}H_{10}O_2$	13.50	0.0745
21	2-Methoxy-4-vinylphenol	150	$C_9H_{10}O_2$	13.90	0.2092
22.	Cyclohexene, 4-ethenyl-4-methyl-3-(1-methylethenyl)-1-(1-methylethyl)-, (3R-trans)-	204	$C_{15}H_{24}$	14.17	0.3675
23	Eugenol	164	$C_{10}H_{12}O_2$	14.59	9.5204
24	Copaene	204	$C_{15}H_{24}$	14.94	0.3259
25.	Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl-	204	$C_{15}H_{24}$	15.13	0.3093
26.	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	204	$C_{15}H_{24}$	15.49	0.1159
27.	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene, [1R-(1R*,4Z,9S*)]-	204	$C_{15}H_{24}$	15.76	4.8802

28	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-	204	$C_{15}H_{24}$	16.00	0.1542
29	à-Caryophyllene	204	$C_{15}H_{24}$	16.35	0.6194
30.	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl- Formula: MW: 202	202	$C_{15}H_{22}$	16.58	0.1080
31.	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]-	204	$C_{15}H_{24}$	16.74	0.2521
32	Eudesma-4(14),11-diene	204	$C_{15}H_{24}$	16.89	0.4408
33.	Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl)-, (S)-á-Bisabolene	204	$C_{15}H_{24}$	16.98	2.2025
34.	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	204	$C_{15}H_{24}$	17.24	0.5270
35	1-Hydroxy-1,7-dimethyl-4-isopropyl-2,7-cyclodecadiene	222	$C_{15}H_{26}O$	17.30	1.4553
36	Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	204	$C_{15}H_{24}$	17.47	0.1924
37	3,7-Cyclodecadiene-1-methanol, à,à,4,8-tetramethyl-, [s-(Z,Z)]	222	$C_{15}H_{26}O$	17.78	3.6269
38.	Bicyclo[4.3.0]nonane, 7-methylene-2,4,4-trimethyl-2-vinyl-	204	$C_{15}H_{24}$	18.27	0.1366
39	(-)-Spathulenol	220	$C_{15}H_{24}O$	18.33	0.1950
40	Caryophyllene oxide	220	$C_{15}H_{24}O$	18.42	0.7127
41.	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-, [1R-(1à,4á,4aá,8aá)]-	222	$C_{15}H_{26}O$	19.29	2.6327
42.	2-Naphthalenemethanol, decahydro-à,à,4a-trimethyl-8-methylene-, [2R-(2à,4aà,8aá)]-	222	$C_{15}H_{26}O$	19.51	0.6217
43	Levomenol	222	$C_{15}H_{26}O$	19.78	2.4712
44.	2-Octenoic acid, 4-isopropylidene-7-methyl-6-methylene-, methyl ester	222	$C_{14}H_{22}O_2$	20.70	0.7816
45	9H-Pyrido[3,4-b]indole, 8-hydroxy-1-methyl-	198	$C_{12}H_{10}N_2O$	20.99	0.5734
46	Bicyclo[3.1.1]hept-3-ene, 2-formylmethyl-4,6,6-trimethyl-	178	$C_{12}H_{18}O$	21.16	0.2403
47.	2-Naphthalenemethanol, 2,3,4,4a,5,6,7,8-octa hydro-à,à,4a,8-tetramethyl-, [2R-(2à,4aá,8á)]-	222	$C_{15}H_{26}O$	21.87	0.2751
48	Naphthalene, decahydro-	138	$C_{10}H_{18}$	23.44	3.9502
49	Piperidine, 1-(1-oxo-3-phenyl-2-propenyl)-	215	$C_{14}H_{17}NO$	27.74	0.6597
50	2-Hydroxy-2-phenylbutyramide	179	$C_{10}H_{13}NO_2$	32.96	0.7099
51.	Benzo[b]cyclopenta[e]pyrane-3-carboxaldehyde, 1,2-dihydro-	198	$C_{13}H_{10}O_2$	35.75	4.4858

52	Furane-2-carbohydrazide, 5-phenylethynyl-	226	$C_{13}H_{10}N_2O_2$	35.95	6.5202
53	o-Anisic acid, 2-adamantyl ester	286	$C_{18}H_{22}O_3$	36.22	17.9390
54	Piperine	285	$C_{17}H_{19}NO_3$	36.64	3.2506
55	Piperine	285	$C_{17}H_{19}NO_3$	41.24	26.6745

CHROMATOGRAPHIC PROFILING BY RP-HPLC :

The RP-HPLC chromatogram (Figure : 2) revealed the presence of 8 compounds (Fig : 2) in the extract under 210 nm. Among the constituents, the active compound with retention time of 4.730 min was Piperine which comprises around 10.963 % among the total constituents at this detection nanometer.



S. No	Reten. Time (min)	Area (mV.s)	Height (mV)	Area (%)	Height (%)	W05 (min)
1	3.157	204.134	29.403	35.4	39.9	0.10
2	3.407	81.259	9.379	14.1	12.7	0.16
3	3.553	129.750	17.140	22.5	23.3	0.12
4	4.310	11.071	1.204	1.9	1.6	0.15
5	4.487	19.059	1.678	3.3	2.3	0.23
6	4.730	63.182	7.243	11.0	9.8	0.13
7	5.163	18.780	2.023	3.3	2.7	0.14
8	5.473	49.110	5.586	8.5	7.6	0.14
	Total	576.345	73.656	100.0	100.0	

Active Fraction (Piperine): R.T - 4.730 min.

Quantitative presence of piperine in the extract based on peak area: 10.963% or 109.63mg/g of extract

Fig. 2 : RP-HPLC Chromatogram and Tabulation of findings

FT-IR SPECTROSCOPY STUDY :

Infra Red spectrum (Figure – 3) was recorded for powdered sample of *P nigrum* using RP-HPLC technique and the data indicates the presence of an amide, ether, alkane as well as aromatic and aliphatic alkene functional groups. All signals were tabulated accordingly in Table - 3 and matched with the predicted spectra for piperine. In this spectral analysis KBr pellet technique was used. Spectrum of the pellet is taken from 4000 cm^{-1} to 400 cm^{-1}.

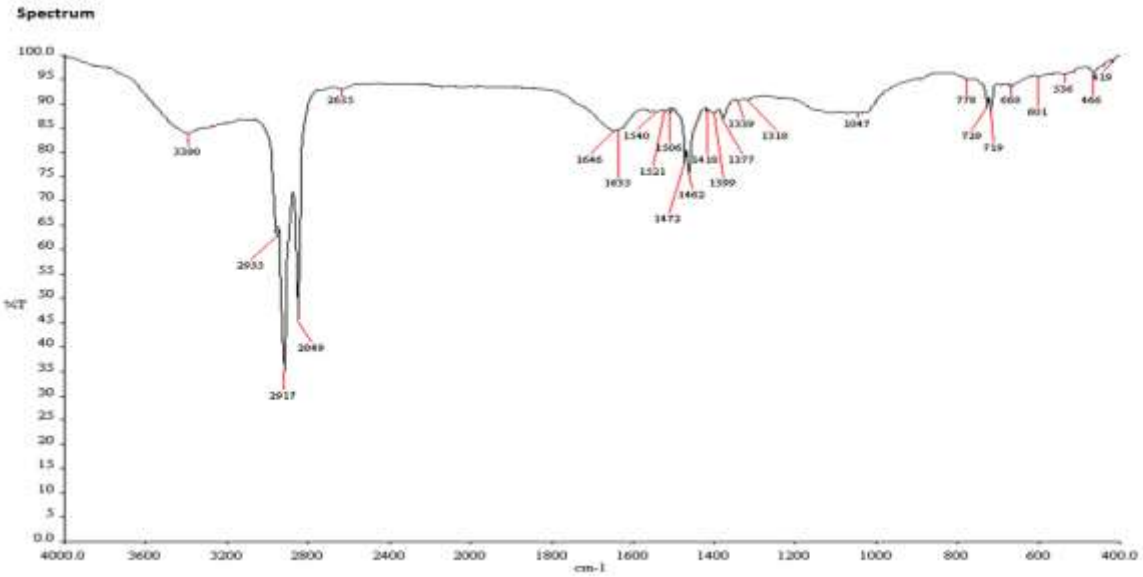


Figure - 3 : FT-IR Spectrum of *P nigrum* Linn.

Table – 3 : FT-IR Interpretation of Spectrum

S. No.	Frequency cm^{-1}	Functional groups
01	3390, 1646, 1635	**CO-N (Amide)**
02	1339,1318, 1047	**R-O-R (Ether)**
03	778, 729, 719	**HC=CH (Alkene – Aliphatic)**
04	1540, 1521, 1506, 1399, 1377	**HC=CH (Alkene – Aromatic)**
05	2955, 2917, 2849, 2635, 1472, 1462	**CH_2 (Alkane)**
06	668-419	**KBr (Standard)**

***In-Silico* Molecular Docking analysis :**

Molecular docking of piperine into the binding site of *Aeromonas hydrophila* aerA protein and the estimation of binding affinity of the ligand is a most important part in the structure based drug designing processes. Piperine shows best possible binding mode against modeled *Aeromonas hydrophila* aerA protein, which is illustrated in Fig. 4. During the molecular docking procedure, the program selects only the best fit active site pockets of the protein with respect to the ligand in order to dock them. The docking program place both the ligand as well as protein in different binding orientations, conformational positions and the lowest energy confirmations which are energetically favorable are evaluated and analyzed for interactions. Free energy of binding interaction (ΔGb) was calculated by AutoDock 4.2 and the binding energy and interacting amino acid residue was found to be -7.2 Kcal/Mol. For all the molecules, binding affinity was characterized by binding energy (ΔG) value. Piperine shows the good binding energy of -7.2 kcal/mol with interacting amino acids like Val 127, Arg 335, Trp 126, Arg 322 and Trp 323. The docking interactions *Aeromonas hydrophila* aerA protein with piperine was shown in the Fig. 5.

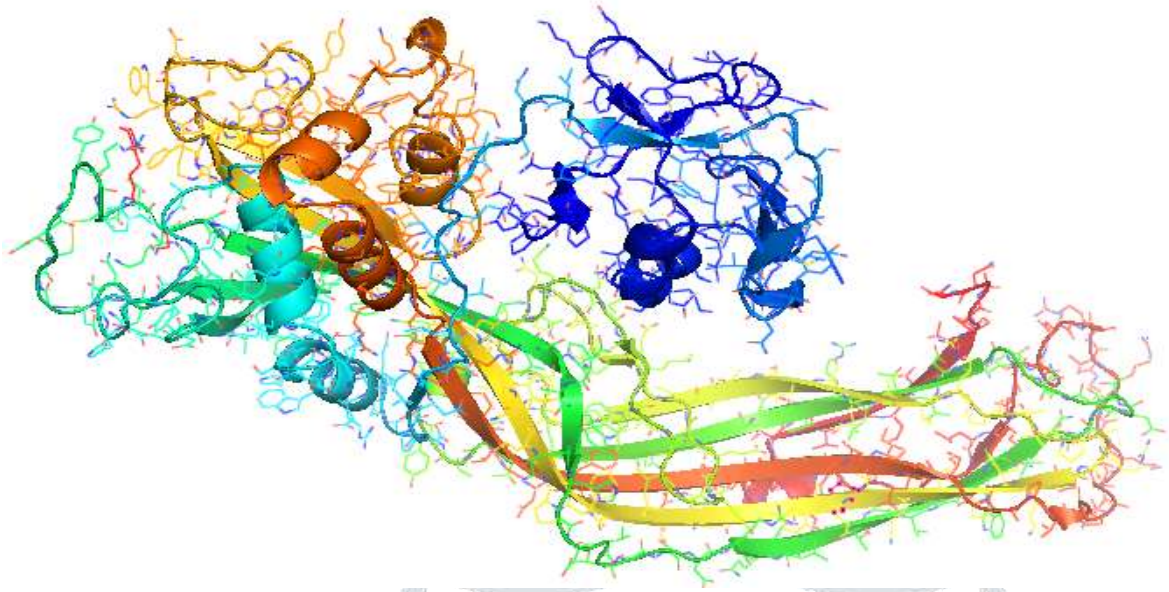


Fig. 4 : MODELLED STRUCTURE OF AEROMONAS HYDROPHILA

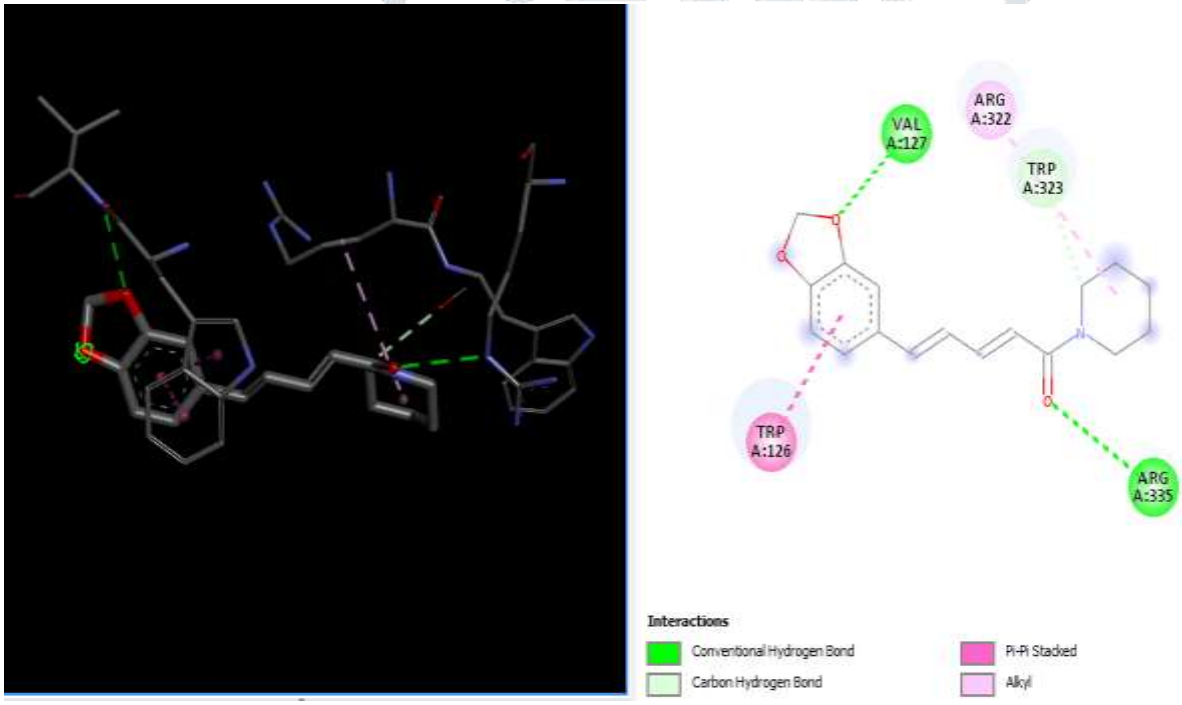


Fig. 5 : DOCKING INTERACTIONS OF aerA PROTEIN WITH PIPERINE

CONCLUSION :

GC/MS spectrum showed the presence of fifty five phytochemical constituents. The high peak compounds in this sample were Piperine, Bicyclo [7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-, [1R-(1R*,4Z,9S*)]-, Eugenol and o-Anisic acid, 2-adamantyl ester. All these compounds were pharmacologically important and they showed the properties such as analgesic, antidiabetic, antibacterial and antifungal activity. RP-HPLC analysis provided a good platform for identification and quantification of piperine present in *P nigrum* seeds. The results of FT-IR spectral analysis confirmed the presence of amide, ether, aliphatic and aromatic alkene and alkane functional groups. Docking the modeled protein (*Aeromonas hydrophila* aerA) with piperine provided insight into the binding and interaction with the enzyme. The examined binding energy was found to be -7.2 kcal/mol. It is hoped that, pharmacological activity testing of

the each phytoconstituents for their individual biological activity will definitely bring fruitful outcome in the development of novel drugs from natural origin.

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