Indian Journal of Chemistry
Vol. 63, July 2024, pp. 692-700
DOI: 10.56042/ijc.v63i7.7567

Therapeutic potentials of characterized isolate from column chromatography by GC-MS and molecular docking of principle active of *Prunus domestica* L.

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Received 27 December 2023; accepted (revised) 28 June 2024

Chromatographic techniques aid in isolating and separating phytoconstituents from natural products, enabling the discovery of innovative compounds of pharmacological and physiological importance. The present study is focused on isolation and identification of the compounds in column chromatography-derived fraction using GC-MS and its biological activities. The ethyl acetate extract of the leaves of *Prunus domestica* L. has been column chromatographed using gradient of ethyl acetate in petroleum ether as solvent. This has yielded a red coloured fraction/band (RCF) (approximately 130 mg), which has further been analysed by GC-MS for the identification of bioactive phytoconstituents. The RCF has been analysed for antioxidant activity by DPPH method which has shown an IC50 value of 43.895 mg/mL. The antibacterial activity of RCF has been assessed by agar well diffusion method against *E. coli* which has shown the zone of inhibition of 17 mm. Stigmast-5-en-3-ol, oleate (31.77%) is identified as the major phytoconstituent and molecular docking of this major constituent has been carried out to observe its interactions with heme oxygenase and DNA gyrase protein chains.

Keywords: *Prunus domestica* L., Column chromatography, GC-MS, Antioxidant activity, Antibacterial activity, Molecular docking

Modern medicine is expanding towards the therapeutically active plant-based medications. These drugs are efficacious, safe and have a broad therapeutic spectrum[1]. Fruits and vegetables are an excellent source of phenolic acids, flavonoids, tannins and nitrogen compounds like alkaloids and amines, in addition to vitamins, terpenoids and other metabolites. All these metabolites possess a high level of antioxidant activity and are associated with a reduced risk of developing chronic diseases. These compounds neutralize or scavenge reactive species *via* hydrogen donation before they harm cells and other biological components, reducing oxidative stress[2].

Plums (*Prunus domestica* L.) are a valuable fruit in terms of functional foods and nutraceuticals. They may aid the body in its fight against a wide range of illnesses[3]. Plums are classified under the Rosaceae family and the *Prunus* genus. The fruits of plum come in an extremely diverse spectrum of sizes, colours, flavours and textures[4]. Plums are distributed all over the world, with the biggest demand being seen in Europe, North America and Japan. The yearly production of plums across the world is roughly 11,000,000 tons[5]. Plums are rich in a variety of polyphenolic compounds. These chemical compounds have the potential to exert many different pharmacological activities, including antibacterial, antioxidant, anti-inflammatory, anticancer, antihyperglycemic, antihyperlipidemic, antihypertensive, antiosteoporosis, laxative and hepatoprotective activities[1,6].

In India, prunes (dried plums) have a long history of use in traditional medicine, particularly in combination with other medicines for the treatment of leukorrhea, irregular menstruation and debility following the loss of a pregnancy. In more recent times, however, prunes have gained popularity as a nutritious food option. High consumption of the dietary fibres found in prunes has been shown in prior research on prunes to reduce levels of low-density lipoprotein (LDL) cholesterol[7]. Plum leaves are seldom utilized in conventional medicine. Data from the scientific literature suggest that leaves are rich in polyphenols, vitamins, pectin and other physiologically active components[1].

Fruits, grains, seeds, nuts and legumes are only a few examples of plant-based foods that may contain hundreds or even thousands of different

phytochemicals (biologically active molecules)[8]. The plant extracts obtained by different extraction processes are generally a complex mixture and consist of numerous types of natural compounds with varying polarity. To achieve a pure bioactive molecule, more separation and purification are required. Their separation remains a formidable obstacle in the process of identifying and characterizing the bioactive compounds[9]. Several separation methods, including thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), paper chromatography (PC), column chromatography (CC), gas chromatography (GC), optimum performance laminar chromatography (OPLC) and high-performance liquid chromatography (HPLC) have been used to separate and purify the bioactive natural compounds. However, CC and TLC are still widely used owing to their ease, cost-effectiveness and accessibility to stationary phases of many types. The identification of the isolated compound is usually done by Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and mass spectrometry (MS)[10].

Since most of the work is done on the fruit and prune (dried fruit) of plums, in the present work, the ethyl acetate extract of the leaves was column chromatographed to yield a red-coloured fraction (RCF) dominated by an ester of steroid stigmast-5-en-3-ol, oleate (31.77%), identified by GC-MS, which was further studied for its antioxidant and antibacterial potential. The molecular docking of the major constituent identified by the GC-MS was also carried out to study inhibitory effect on HO-1 and DNA gyrase enzymes.

Experimental Section

Reagents and Chemicals

Petroleum ether (SD fine chem), diethyl ether (SD fine chem), ethyl acetate (SD fine chem), methanol (Rankem), and silica gel for column chromatography of mesh 100-200 (Merck). All the other chemicals and reagents used in the analysis were either HPLC or analytical grade.

Authentication of the Plant

The Botanical Survey of India (BSI), Dehradun, identified and verified the plant under accession number 374.

Collection of the Plant Material

The leaves of the plants were collected in May 2021 from the local orchards situated in Haridwar.

Extraction

For the extraction, soxhlet extraction method was used. In the thimble of the soxhlet extractor, 200 gm of powdered plant material was placed. Three solvents petroleum ether, diethyl ether and ethyl acetate were used for extraction in increasing order of polarity. Each solvent underwent about 72 cycles of siphoning, or the extraction was prolonged until the siphoning tube became colourless. After extraction, the extracts from various solvents were concentrated under reduced pressure using a rotary evaporator, then stored in a refrigerator for further analysis. The ethyl acetate extract was chosen for the column chromatography to obtain the bioactive phytoconstituents.

Column Chromatography

25 gm of ethyl acetate extract from the leaves was dissolved in methanol and added to 50 gm of silica for column chromatography of mesh size 100-200. The resulting mixture was dried in the oven at 50°C till the silica became free to flow. A column with a length of 42 inches and a diameter of 1.5 inches was used for the column chromatography. The column was packed with wet packing using silica slurry in petroleum ether. After packing, the column was loaded with the silica containing the ethyl acetate extract. Initially, the column was run with petroleum ether for a minimum four column volume then the polarity was increased to 1% v/v with ethyl acetate in petroleum ether. The red-coloured band/fraction (RCF = 130 mg) from the column was collected in a conical flask and further analysed by gas chromatography-mass spectroscopy (GC-MS) (Fig. 1).

Identification of the Bioactive Phytoconstituents Using Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was performed according to Mahmood *et al.*, (2009) with some modifications[11]. GC-MS (Shimadzu-TQ8040), mobile phase He at 1 mL/min, SH-Rxi-5Sil MS capillary column (Stationary phase: 5% diphenyl–95% dimethyl polysiloxane, length: 30 m, inner diameter ID: 0.25 mm, film thickness df: 0.25 μm), split ratio 1:10, injection temperature 280°C, the column oven temperature was raised from 80 to 280°C at rate

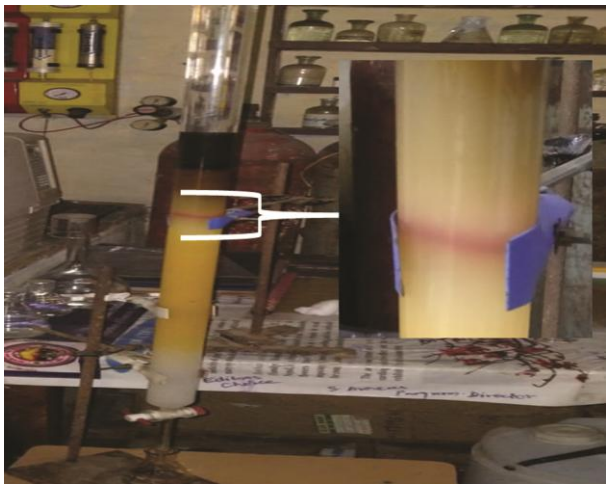


Fig. 1 — Column Chromatography of ethyl acetate extract of leaves

5°C/min. MS conditions were as follows: Ion source was kept at 220°C with detector voltage 0.7 kV, m/z was in the range of 40 to 500 and scan time was set at 0.30 seconds.

Antioxidant Activity (DPPH Method)

The antioxidant activity was assessed according to Brand-William *et al.*, (1995) with a little alteration[12]. Briefly, 3 mL of 0.004% working DPPH solution in methanol was mixed with 1 mL sample solution at varying concentrations in DMSO. The resultant mixture was incubated at room temperature for 30 minutes. After that, the absorbance of the resultant mixture was measured at 517 nm using a double-beam UV-Vis spectrophotometer (Systronics 2205). Ascorbic acid was used as standard. The blank experiment was also conducted in similar manner. The change in the colour of the DPPH solution from purple to yellowish is indicative of the antioxidant capacity of the sample solution. The % DPPH scavenging (or inhibition) was calculated by the following formula:

$$\%\ inhibition = \frac{Absorbance\ of\ the\ blank - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ blank} \times 100$$

IC50 (concentration required to scavenge the 50% DPPH radical) value of RCF was calculated by a graphical method by plotting the % inhibition against concentration. Another important parameter, antioxidant activity index (AAI) was calculated by using the following equation[13]:

$$Antioxidant\ activity\ index\ (AAI) = \frac{Final\ DPPH\ concentration\ (\mu g\ per\ ml)}{IC50\ value\ (\mu g\ per\ ml)}$$

Antibacterial Activity

The agar well diffusion method was used to evaluate the antibacterial activity[14]. The test microorganism was grown on Muller Hinton Agar (MHA) and Muller Hinton Broth. The MHA that had been sterilized was put into petri plates and left for a while to solidify. The fresh overnight culture of microbial strain (*E. coli*) having turbidity equivalent to Mac Farland standard (0.5) was swabbed uniformly on the MHA plates. A cork borer was then used to pierce the well. Then 200 µl of RCF at a concentration of 100 mg/mL in DMSO was poured into well and allowed to incubate at 37°C for 18 to 24 hours and measured the inhibition zone. Standard discs of commercially available antibiotics (chloramphenicol and gentamicin) were used as standard.

Molecular Docking of the Major Phytoconstituent

The method described by Rizvi *et al.*, (2013) with a few alterations was adopted to perform the molecular docking[15]. The procedure followed is as below:

- Selection of Target Protein: The crystal structure of human heme oxygenase 1 (HO-1) in complex with its substrate heme, crystal form B having PDB ID as 1N3U with resolution 2.58 Å and the crystal structure of DNA gyrase (*E. coli* 24kDa domain) in complex with clorobiocin having PDB ID as 1KZN with resolution 2.30 Å were assessed from Protein Data Bank (www.rsc.org). These proteins were further processed to remove hetatoms, water molecules and additional chains (if any). The addition of polar hydrogen, kollmann charges was done in DSV 2021 and Autodock 4.2 software to optimize the conditions.
- Designing of Ligand and Molecular Docking: The ligand, *i.e.*, stigmast-5-en-3-ol, oleate was assessed from www.molinstincts.com, which was further converted in PDB and PDBQT format by DSV 2021 software. The final docking was performed in Autodock4.2 software.

Results and Discussion

Column Chromatography and Gas Chromatography-Mass Spectroscopy

A total of 32 bioactive compounds are identified in RCF. The identification of the compounds was done using the matching of mass spectral library search program which are shown in the Table 1. The amount of the compound was determined by the relative peak area of the compound in chromatogram (Fig. 2).

Table 1 — Detection of compounds in RCF by GC-MS

S. No.	Compound name	%	Mm	R_t
1.	(6E,10E,14E,18E)-2,6,10,15,19,23-Hexamethyl-1,6,10,14,18,22-tetracosahexaene-3-ol	0.39	426	32.350
2.	1,4-Diaza-9-oxaspiro[5.5]undecane, 8-ethyl-8-methyl-	15.01	198	33.535, 35.125, 36.655, 40.135, 42.165
3.	13-Methyl-Z-14-nonacosene	0.92	420	49.670
4.	1-Dodecanol	0.50	186	8.045
5.	1-Heptacosanol	0.58	396	26.375
6.	1-Hexacosanol	1.44	382	30.055, 39.915, 45.165
7.	1-Nonadecene	1.88	266	17.909, 22.345
8.	2-Methylpentacosane	0.73	366	36.080
9.	2-Methyltetracosane	0.67	352	34.525
10.	3-Methylhexacosane	0.83	380	37.770
11.	3-Methylpentacosane	0.83	366	37.605
12.	7-Methyl-1,5-diazacyclotetradecane	2.63	212	38.235
13.	9-Anthraldehyde hydrazone	5.80	220	36.335
14.	Bicyclogermacrene	0.74	204	24.765
15.	Cyclohexane, 1,5-dimethyl-2,3-divinyl-	2.99	164	29.555
16.	Dodecane, 2,6,11-trimethyl-	0.46	212	8.247
17.	E,Z-1,3,12-Nonadecatriene	1.39	262	29.440
18.	Eicosane	0.35	282	26.490
19.	Hexacontane	9.19	842	39.365, 39.570, 40.505, 41.520, 41.780, 45.380, 48.440, 49.075
20.	Hexadecane	0.58	226	13.267
21.	N-[6-Cyclododecylaminohexyl]aziridine	5.12	308	30.155, 31.880
22.	Neocembrene	0.36	93.10	26.105
23.	n-Heptadecanol	0.86	256	13.072
24.	Phenol, 3,5-bis(1,1-dimethylethyl)-	2.69	206	16.120
25.	Phytyl palmitate	0.86	534	30.385
26.	Pregna-5,16-dien-20-one, 3-(acetyloxy)-, (3β)-	1.29	356	48.730
27.	Squalene	2.27	410	32.475, 34.395, 40.360
28.	Stigmast-5-en-3-ol, oleate	31.77	678	47.710
29.	Succinic acid, hex-4-yn-3-yl tetradec-3-en-1-yl ester	0.61	392	48.260
30.	Tetracosamethyl-cyclododecasiloxane	3.51	888	36.857
31.	Tetracosane	0.41	338	41.375
32.	Z-5-Nonadecene	1.60	266	28.360

Where: Mm= Molecular mass of compound and Rt = Retention time (in minutes).

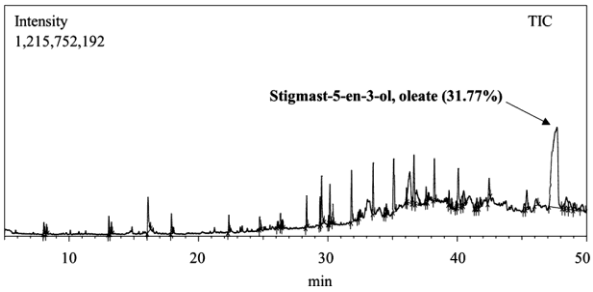


Fig. 2 — Total ion chromatogram (TIC) of isolated RCF

Stigmast-5-en-3-ol, oleate (31.77%) is found to be the most abundant compound which is an ester of the steroid. Stigmast-5-en-3-ol, oleate has been reported for its anti-cholesterol activity[16]. Stigmast-3-en-5-ol, is also an antidiabetic agent[17]. Three other compounds identified with an amount >5% are 1,4-diaza-9-oxaspiro[5.5]undecane, 8-ethyl-8-methyl- (15.01%), hexacontane (9.19%) and 9-anthraldehyde hydrazone (5.80%). Hexacontane is high molecular weight hydrocarbon with waxy solid consistency. It is employed in the extraction of Arbutus unedo, a strawberry tree[18]. In addition to this, 9-anthraldehyde hydrazone is a derivative of hydrazone. Hydrazones are known to show a wide variety of biological activities including antimicrobial, anticonvulsant, antidepressant, anti-inflammatory, antitubercular, antiviral, cardio protective, analgesic, antiplatelet, antimalarial, anticancer and antifungal activities[19].

Antioxidant Activity

The DPPH approach is one of the simplest and most widely used method for the determination of the antioxidant activity. The unpaired valence electron on the nitrogen atom in the DPPH radical is reduced by the hydrogen atom from the antioxidant molecule. As a result, DPPH-H hydrazine is produced[20]. Spectrophotometric measurements may be used to determine the amount of quenched DPPH radicals by analysing the decline in absorbance between 515 and 520 nm[21]. The quantity of antioxidants required to reduce the initial concentration of DPPH free radicals by 50% is used to represent the antioxidant activity and is called as IC_{50} (or EC_{50}). The results of the antioxidant activity are shown in Table 2. The IC_{50} value of the RCF is found to be 43.895 mg/mL. Vitamin C, *i.e.*, ascorbic acid was used as standard which has shown an IC_{50} value of 387.718 µg/mL. Fig. 3 demonstrates the graphical representation of the RCF and standard. The graphs are plotted between concentration (x-axis) and % inhibition (y-axis). In addition to IC_{50} another important parameter antioxidant activity index (AAI) is also calculated. The AAI of RCF is found to be 9.094×10^{-4}, while for ascorbic acid AAI is found to be 1031.677×10^{-4}.

Antibacterial Activity

The results of the antibacterial activity of the RCF are shown in Table 3. Commercially available discs of the antibiotic drugs (chloramphenicol 25 µg and gentamicin 30 µg) were used as standard. The antibacterial activity was carried out against the *E. coli* which is a gram-negative bacterium. Gram negative bacteria have a thin cell wall formed of peptidoglycan. They have an exterior membrane that contains lipids and is separated from the cell wall by the periplasmic space in addition to an inner membrane which comprises periplasmic enzymes and binding proteins[22,23]. The results are expressed in terms of zone of inhibition (in mm). Fig. 4 and 5 show the petri plates and histogram of RCF and standard drugs respectively. The RCF has shown a zone of inhibition of 17 mm while chloramphenicol

Table 2 — Antioxidant activity of RCF by DPPH inhibitory assay

Name	IC_{50} (or EC_{50})	Antioxidant Activity Index
RCF	43.895 mg/mL	9.094×10^{-4}
Ascorbic Acid (Standard)	387.718 µg/mL	1031.677×10^{-4}

Table 3 — Antibacterial activity of RCF

Name	Zone of Inhibition (mm)
RCF (200 µL of 100 mg/mL)	17
Chloramphenicol (25 mcg)	25
Gentamicin (30 mcg)	28

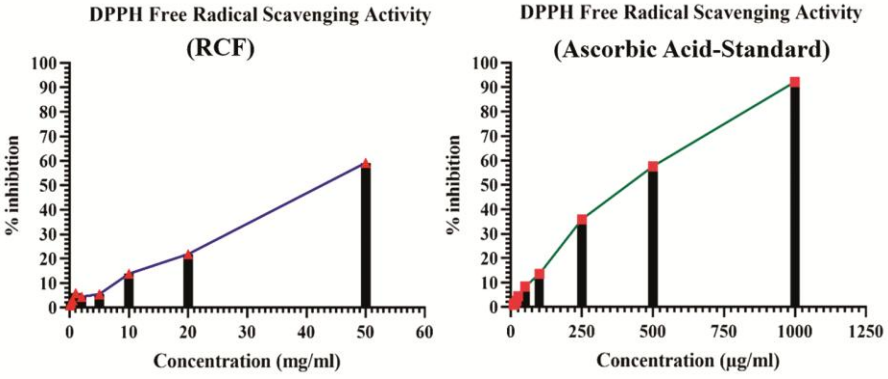


Fig. 3 — Variation of % inhibition of DPPH against concentration

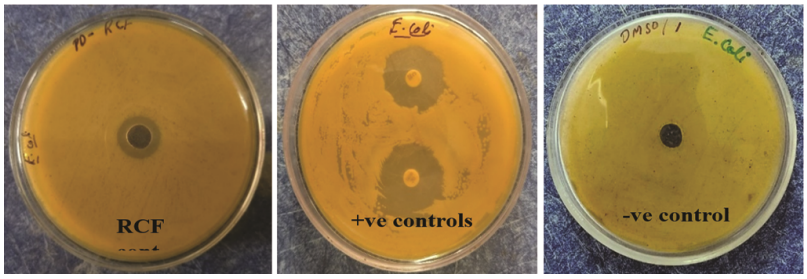


Fig. 4 — Petri-plates representing Antibacterial Activity of RCF, Positive and negative controls

and gentamicin have displayed the zones of inhibition of 25 mm and 28 mm, respectively. Since the plant extracts always demonstrate a complex mixture of the secondary metabolites therefore the role of the other minor metabolites cannot be nullified and thus it is difficult to reduce the antibacterial effect of the RCF to some major active metabolites, other minor active metabolites must have some contribution towards the activity[24]. But the major contribution in the activity is most probably due to the major metabolites as identified by GC-MS.

Molecular Docking

Heme oxygenase-1 (HO-1) is a cellular stress protein involved in the oxidative catabolism of heme, which results in the synthesis of biliverdin (BV), free iron and carbon monoxide (CO). Whereby, the produced BV is promptly changed to the potent antioxidant bilirubin (BR), which is subsequently turned back into BV by interacting with ROS and neutralising them. As a result, HO-1 has the capability to regulate oxidative and inflammatory processes, which contributes to its efficacy in managing metabolic illnesses and makes it a target for research work[25]. DNA gyrase is a topoisomerase enzyme that catalyses changes in the topology of DNA. Since DNA gyrase is found in all bacteria except higher eukaryotes, it is a therapeutic target for antibacterial research[26]. The results of the molecular docking are shown in Table 4. Fig. 6 and 7 represent the 3D and 2D visualization of interaction of the stigmast-5-en-3-ol, oleate, ascorbic acid and chloramphenicol, with HO-1 and DNA gyrase. Stigmast-5-en-3-ol, oleate has shown good docking score (best out of 10 conformations) of –7.44 and –4.13 kcal/mol in both HO-1 and DNA gyrase respectively. These values are indicative that the stigmast-5-en-3-ol, oleate is fitted nicely in the active pocket of the targeted protein. Here the docking analysis justifies the good results of antioxidant and antibacterial activity. The ascorbic acid and gentamicin were used as inhibitors for HO-1 and DNA gyrase in control experiments which showed the docking score (–3.23 kcal/mL) and (–4.58 kcal/mL) respectively. Thus, the binding models shown here imply that stigmast-5-en-3-ol, oleate is a good inhibitor of HO-1 and DNA gyrase and some key structural point to be considered in future optimization.

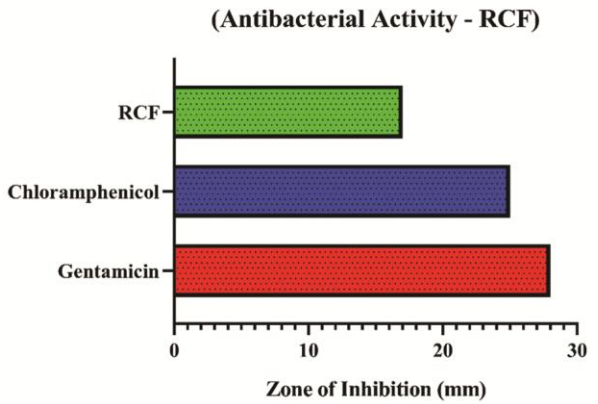


Fig. 5 — Histogram of Antibacterial Activity of RCF and Standards

Table 4 — Docking energy and interaction sites between target and ligand

Ligand	Protein (PDB ID)	Co-ordinates of central grid point of map and grid points	Docking energy and binding constant Ki	Type of amino acid interacted with ligand
Stigmast-5-en-3-ol, oleate	Heme oxygenase (1N3U)	(x, y, z) = (19.968, 19.235, –29.945) and (x, y, z) = (60, 60, 60)	–7.44 Kcal/mol and 3.49 µM	Val50, Met34, Leu147, Phe166, Ser142, Lys179, Leu138, Tyr134, Phe207, His25
	DNA gyrase (1KZN)	(x,y,z) = (19.540, 19.167, 43.283) and (x,y,z) = (60, 60, 60)	–4.13 Kcal/mol and 931.47 µM	Val71, Val167, Ala47, Ile78, Ile90, Val120, Val43
Ascorbic acid	Heme oxygenase (1N3U)	(x, y, z) = (19.968, 19.235, –29.945) and (x, y, z) = (60, 60, 60)	–3.23 Kcal/mol and 4.28 mM	Asp140, Asn210, Arg136
Chloramphenicol	DNA gyrase (1KZN)	(x,y,z) = (19.540, 19.167, 43.283) and (x,y,z) = (60, 60, 60)	–4.58 Kcal/mol and 440.09 µM	Thr165, Asp73, Glu50, Ala47, Asn46, Pro79, Ile78

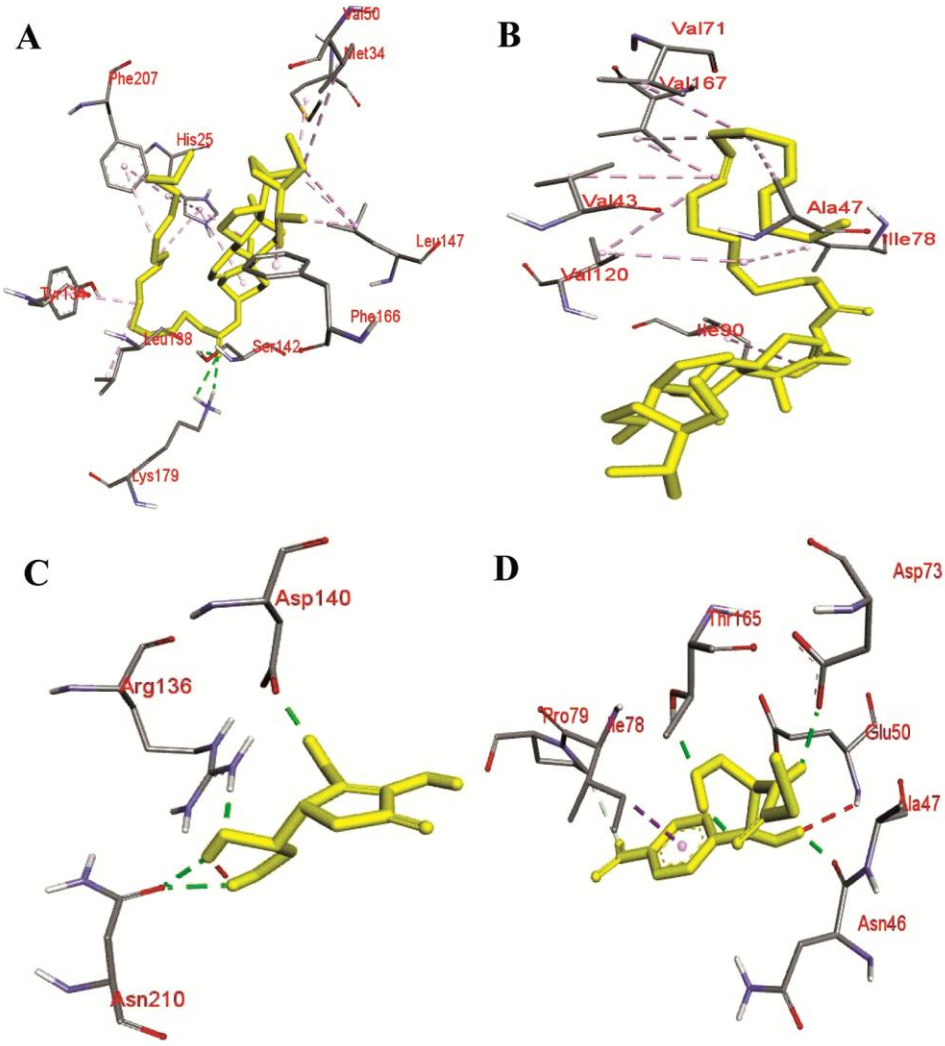
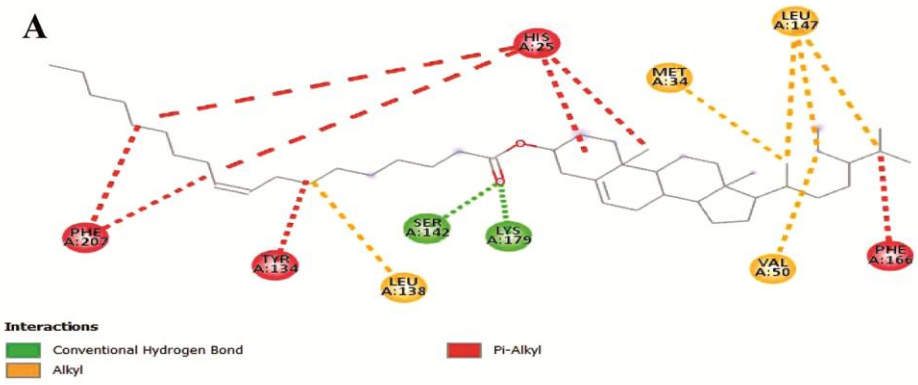


Fig. 6 — A and C represent the 3D visualization of molecular interaction of heme oxygenase-1 with stigmast-5-en-3-ol, oleate and ascorbic acid respectively. B and D represent the 3D visualization of molecular interaction of DNA gyrase with stigmast-5-en-3-ol, oleate and chloramphenicol respectively



(*Contd*. Fig. 7)

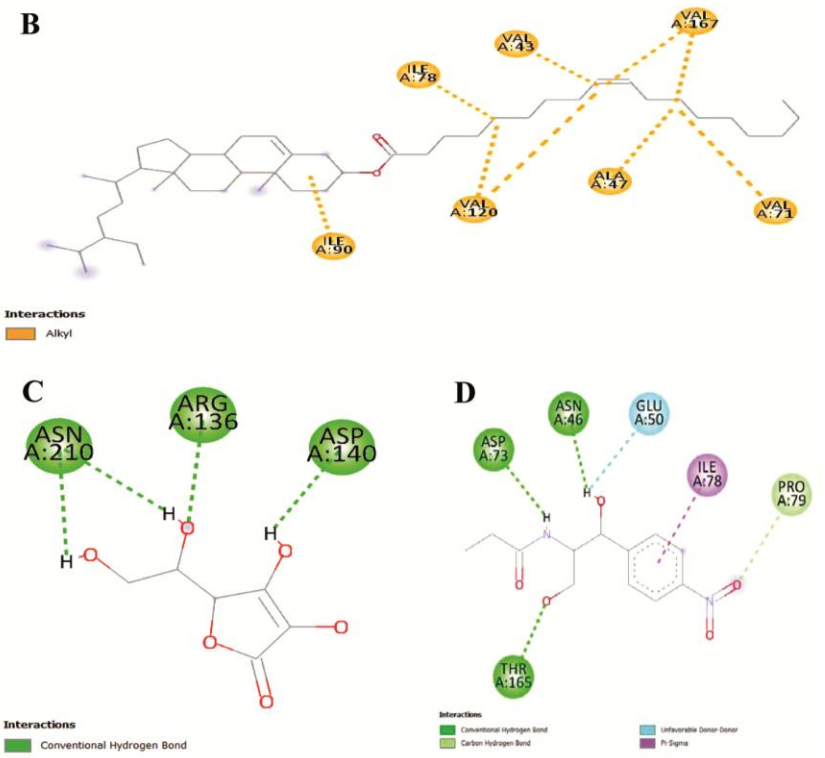


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Fig. 7 — A and C represent the 2D visualization of molecular interaction of heme oxygenase-1 with stigmast-5-en-3-ol, oleate and ascorbic acid respectively. B and D represent the 2D visualization of molecular interaction of DNA gyrase with stigmast-5-en-3-ol, oleate and chloramphenicol respectively

Conclusions

Column chromatography is one of the most important techniques widely used for the separation of the individual constituent from any mixture of compounds. Since the plant extract being the complex mixture of naturally occurring secondary metabolites, the use of the column chromatography becomes extremely crucial. While GC-MS is also a kind of separation technique carrying mass detector which allows the identification of the constituents of a mixture. The fraction obtained by column chromatography was subjected to GC-MS and resulted in the tentative identification of 32 compounds with stigmast-5-en-3-ol, oleate as dominating compound. It is an ester of steroid. The fraction was further assayed for antioxidant and antibacterial activity. Evaluating antibacterial and antioxidant activity supports in the discovery and development of novel antimicrobial medicines, allowing for alternative therapies for infectious illnesses, ageing and oxidative stress. Docking analysis shows the binding orientation of stigmast-5-en-3-ol, oleate in the HO-1 and DNA gyrase binding pockets. The stigmast-5-en-3-ol, oleate interactions with HO-1 and DNA gyrase proteins suggested that it might operate as an efficient inhibitor against oxidative and bacterial stress.

Acknowledgements

Kishan is extremely thankful to CSIR, PUSA, New Delhi for providing SRF fellowship. The authors are extremely grateful to Mr. Sahil Kumar, Research Scholar, Department of Botany and Microbiology, GK(DU), Haridwar, for his valuable assistance in carrying out the antibacterial activity.

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