Asian Journal of Chemistry; Vol. 34, No. 2 (2022), 342-354

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23521

In silico, ADMET and Docking Analysis for the Compounds of Chloroform Extract of *Tinospora cardifolia* (Wild.) Identified by GC-MS and Spectral Analysis for Antidiabetic and Anti-Inflammatory Activity

D. SENTHIL KUMAR[1], D. KARTHIKEYAN[2] and BISWABARA ROY[3,*]

[1]Department of Pharmacy, Faculty of Engineering & Technology, Annamalai University, Annamalai Nagar-608002, India
[2]SNS College of Pharmacy and Health Sciences, SNS Kalvi Nagar, Kurumbalayam, Saravanampatti, Coimbatore-641035, India
[3]Srikrupa Institute of Pharmaceutical Sciences, Kondapakka, Velikatta, Siddipet-502277, India

*Corresponding author: E-mail: bishwabara1980@gmail.com

Received: 6 September 2021; *Accepted*: 1 November 2021; *Published online*: 11 January 2022; AJC-20657

The present study was aimed to phytochemical and GC-MS analysis for chloroform extract of *Tinospora cardifolia*. The structure of the compounds was further confirmed by UV-spectroscopy and FTIR study. The *in silico* study like molecular, physico-chemical and drug likeliness property was carried out by computational approaches for the identified molecules. Further toxicity potential and pharmacokinetic profile were also determined. The study was carried out using OSIRIS data warrior and Swiss ADME tools. The docking analysis was carried out for the antidiabetic and anti-inflammatory profiles. The compounds were targeted for α-glucosidase, peroxisome proliferator-activated receptor, glucose transporter-1, cyclo-oxygenase-1 & 2 inhibitions. There were around 12 compounds identified by GC-MS analysis. All the compounds exhibited moderate to good drug likeliness and pharmacokinetic potentials. The molecules showed a good bioactivity score against enzyme receptors. The ADMET prediction showed PGP and CYP-inhibitory effects with the least toxic profile. The docking analysis showed strong binding affinity of [1*S*-(1α,3aα,4α,6aα)]-1*H*,3*H*-furo[3,4-*c*]furan tetrahydrophenyl (molecule-7) on targeted proteins under investigation.

Keywords: *Tinospora cardifolia*, Chloroform extract, Drug likeliness, Docking analysis, Anti-diabetic, Anti-inflammatory activity.

INTRODUCTION

Phytomedicines are herbal formulations made up of herbal extracts individually or in combinations used as medicines or cosmetics due to a wide range of therapeutic effects [1]. Herbal medicines are lost their popularity due to the increased use of allopathic medicines and their first effects [2]. But traditional medicines are using for more than 2000 years when the allopathic system was not started [3]. Attention is being focused on the investigation of the efficacy of plant-based drugs used in traditional medicine because they are economic, have few side effects and according to WHO, about 80% of the world population rely mainly on herbal remedies [4].

Guduchi or Amritha scientifically called *Tinospora cardifolia* (Willd.) Miers ex Hook. f. & Thoms. It is widely distributed in different parts of India and China [5]. Around 1000 tonnes plants consume throughout the year in the form of medicines [6]. *T. cardifolia* is used in the form of tonics to treat different types of critical illnesses like jaundice, arthritis, diabetes and different types of skin diseases [7-9]. The major active constituent found in *T. cardifolia* are alkaloids, furano diterpenoids, clerodane norditerpenoids, sesquiterpenoids, phenolics, lignans, sterols, aliphatic compounds, polysaccharides, essential oil and fatty acids [10,11]. The whole plant possesses hepatoprotective, antiulcer and antioxidant properties, whereas the stems showed hepatoprotective, antipyretic, cytotoxic, antidiabetic and immunomodulatory activities [12-17]. Dried fruits are used for jaundice and rheumatism, whereas the leaves are used to treat diabetes [18], and the roots are employed for their powerful emetic, antistress, antioxidant, antiulcer and hypoglycemic properties as well as for the treatment of visceral obstructions [19-23].

In GC-MS analysis, 40 compounds (15 compounds from chloroform extract, 14 compounds from methanol extract and

11 compounds from petroleum ether extract) were detected in the *T. cardifolia* [24], while 45 numbers of phytochemicals were identified in ethyl acetate extract [25]. In rational drug design, major step is the identification and characterization of the bioactive molecules using advanced spectroscopic techniques like X-ray crystallography and nuclear magnetic resonance (NMR). The spectroscopy provides stereochemical information of molecules with the initiation of the structure-based drug design (SBDD) process. The application of insilico drug design is commonly based on background experimental information and computational methodologies [26].

Structure-based drug design describes the specificity and affinity of ligands with specifically targeted proteins [27]. The compound having high binding affinity and specificity is considered a biologically active molecule in respect to specific disease [28]. The foremost widely approaches used are molecular docking, molecular dynamics (MD), fragment based drug design (FBDD) and pharmacophore modeling are referred to because of the commonest computational SBDD methods [27]. *In silico* approaches utilized in ligand-based drug design. It predicts molecular property, physico-chemical property, drug likeliness and ADMET prediction. The foremost widely used approach in ligand-based drug design (LBDD) is ligand chemical similarity, binding affinity and physico-chemical property with standard molecules. The other is pharmacophore mapping and quantitative structure-activity relationship (QSAR) [29].

The simulation of a biomolecular interaction is often achieved by molecular docking. It provides information regarding the affinity of every ligand [30]. The compounds with high relative molecular mass exhibit unsatisfied pharmacokinetic properties, because of poor solubility. A fragment-based drug design (FBDD) approach will be applied to overcome this problem. It is predicated on the identification of the molecules based on Lipinski rule five [31,32]. Present study was aimed to perform web and software-based SBDD, FBDD and LBDD design of the compounds present in the chloroform extract of *Tinospora cardifolia*. The presence of the compounds was determined by spectral and GC-MS analysis. Based on the literature present study, it was also aimed to target specific binding proteins for the compounds of *Tinospora cardifolia* responsible for antidiabetic and anti-inflammatory activities.

EXPERIMENTAL

***Tinospora cardifolia* extract:** The hydroalcoholic extract of *Tinospora cardifolia* was purchased from Herbal creation, Nainital, India. Approximately 100 g of hydroalcoholic extract was suspended in 250 mL of petroleum ether to remove fatty components. Remove the petroleum ether and air-dried. To the dried extract, added 250 mL of chloroform and kept for 24 h. The resulting extracts were evaporated in the rotary flash evaporator to remove excess chloroform. The extract was air dried in a desiccator and stored in an airtight glass container. The resulting extract was used for further analysis.

Phytochemical analysis: The alkaloids, glycosides, steroids, phenolics, aliphatic compounds, polysaccharides, furono diterpene glucoside, protein, calcium and phosphorus are major active chemical constituents of *T. cardifolia*. The hydroalcoholic and chloroform extracts were taken for different phytochemical analysis as per literature procedure [33,34].

GC-MS full scan analysis: The chloroform extract of *T. cordifolia* was subjected to GC-MS full scan analysis. Accurately weighed 50 mg of chloroform extract was dissolved in 100 mL of HPLC grade methanol. The resulting solution was further diluted to 10 mL to get the desired concentration of 30 µg/mL and used for analysis.

The separated compounds were identified by comparing their mass spectra with the mass spectral data of the compounds present in the NIST library data base.

Determination of wavelength: Accurately weighed 100 mg of chloroform extract and transferred into a 100 mL volumetric flask, dilute to 100 mL with phosphate buffer (pH 6.8). From the above solution taken 10 mL of the solution into a100 mL volumetric flask dilute it to volume with diluents and mixed well (concentration: about 100 µg/mL). From the above stock solutions taken 3 mL of the solution to 10 mL of volumetric flask and further diluted to 10 mL to get desired concentration of 30 µg/mL and used for analysis. The solution was scanned from 200-400 nm, the instrument was scanned in spectrum mode and determine the absorbance. The study was carried out in triplicate.

FTIR analysis: The FTIR analysis was carried out by Bruker (3000 Hyperion Microscope with Vertex 80 FTIR System), Germany associated with Micro ATR, Grazing angle. Approximately 1 mg of extract was placed on the sampling plate and was scanned at 4000-450 cm^{-1}, with a spectral resolution of FTIR 0.2 cm^{-1}.

Preparation of structure: The structure of the titled compound was prepared by Chemdraw ultra 12.0.2. SMILES notations of the title compounds were obtained by using ACD labs Chemsketch version 12.0. The structures were converted to mol2 by using Chem 3D pro 12.

Molecular, physico-chemical property and toxicity potential of compounds: The smile notation of compounds was entered in Osiris data warrior software and calculated molecular properties like shape index, molecular flexibility, molecular complexity of the scanned compounds found in GC-MS analysis. Similarly, physico-chemical properties such as molecular weight, partition coefficient (cLog P), water solubility in moles/ liter (cLogS), hydrogen bond acceptors and donors, total surface area, relative polar surface area, topological polar surface area (TPSA) and violations of Lipinski's rule of five were calculated to evaluate the drug likeliness of the compounds.

The toxicity profile like mutagenic, tumorigenic, reproductive effective, irritant property was also calculated. Fraction Csp3 and molar refractive index was calculated using the Swiss ADME online tool http://www.swissadme.ch/index.php. The molecular, physico-chemical property and toxicity potential of the compounds were compared with the standard drugs. The absorption percentage (% Abs) was also determined by using the following formula:

$$\text{Absorbance (\%)} = 109 - (0.345 \times \text{TPSA})$$

Drug likeliness and bioactivity score: SMILES notations of the molecules were placed in the online tool Swiss ADME

(http://www.swissadme.ch/index.php) to predict drug likeliness properties like Lipinski, Ghose, Veber, Egan, Muegge, Bioavailability Score. Molinspiration software version 2011.06 (www.molinspiration.com) was used to calculate the score for drug targets including enzymes and nuclear receptors, kinase inhibitors, GPCR ligands and ion channel modulators. The bioactivity rader of molecules and standards was prepared using the Swiss ADME tool.

Pharmacokinetic potential: The pharmacokinetic potential of the compounds was determined by the online tool to Swiss ADME (http://www.swissadme.ch/index.php). The pharmacokinetic properties like GI absorption, BBB permeant, PGP substrate, CYP1A2 inhibitor, CYP2C19 inhibitor, CYP2C9 inhibitor, CYP2D6 inhibitor, CYP3A4 inhibitor, Log Kp (skin permeation) was also calculated. Based on the values determined boiled egg diagram was prepared using the Swiss ADME tool.

Docking analysis: Docking analysis of the molecules was carried out using Swiss dock (http://www.swissdock.ch/docking) and UCSF Chimera 1.5.3 was used for interactive visualization and analysis of molecular structures and related data, including density maps, trajectories, and sequence alignments. The targeted proteins 5zcb (α-glucosidase), 5ycp (Human PPARgamma ligand-binding domain complexed with Rosiglitazone), 4pyp (crystal structure of the human glucose transporter GLUT1), 1eqg (The COX-1 complexed with ibuprofen), 3ln1 (structure of celecoxib bound at the COX-2 active site) was collected from RSCB protein data bank in pdb format. The molecules were converted to Mole2 file format.

The proteins and molecules was submitted to the Swiss dock server for flexible docking. Docking analysis was carried out by UCSF Chimera 1.5.3 to analyze binding score, binging pose and binding residue. Out of 250 clusters least one is considered as best binding score.

RESULTS AND DISCUSSION

Though different types of pharmacological activity were reported for the selected plant. The present study was focused on the antidiabetic and anti-inflammatory activities of the plant extract. The structure-based flexible docking analysis was carried out for compounds found in the extract. The main aim behind the study was to find out the molecules which one is selectively responsible for antidiabetic and anti-inflammatory activities.

Phytochemical analysis: The maximum percentage yield for chloroform extract of *Tinospora cardifolia* was found to be 22.8%. The phytochemical analysis was carried out for the extract as per the literature procedure. The presence of alkaloids, flavanoid, steroid, phenolic compound, lignin, terpenoids and aliphatic compounds in chloroform extract of *Tinospora cardifolia* is detected (Table-1).

TABLE-1
PHYTOCHEMICAL ANALYSIS

Phyto constituents	Hydroalcoholic extract	Chloroform extract
Alkaloid	Positive	Positive
Steroid	Positive	Positive
Flavanoid	Positive	Positive
Phenol	Positive	Positive
Lignan	Positive	Positive
Tannin	Positive	Negative
Saponin	Positive	Negative
Carbohydrates	Positive	Negative
Vitamins	Positive	Negative
Terpenoid	Positive	Positive
Aliphatic compounds	Positive	Positive

GC-MS analysis: The GC-MS chromatogram is shown in Fig. 1 and the results are tabulated in Table-2. A total of 12

TABLE-2
GC-MS ANALYSIS FOR CHLOROFORM EXTRACT OF *Tinospora cardifolia*

Molecule	Time (min)	Percentage of peak area	m.w.	m.f.	Name of compound[a]	Probability (%)[a]
1	18.5037	9.555884938	180	$C_{10}H_{12}O_3$	4-[(1*E*)-3-hydroxy-1-propenyl]-2-methoxy phenol	73.9
2	21.5213	5.676235595	250	$C_{14}H_{22}N_2O_2$	5,10-Diethoxy-2,3,7,8-tetrahydro-1*H*,6*H*-dipyrrolo[1,2a,1',2'd]pyrazine	68.9
3	22.194	6.060772313	210	$C_{11}H_{14}O_4$	2,5-Dimethoxy-benzenemethanol acetate	14.2
4	30.001	6.904304091	192	$C_{13}H_{20}O$	1*H*-2-indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl	18.4
5	30.9136	9.995264399	250	$C_{16}H_{26}O_2$	3-Cyclopentyl-6-hydroxy-6-methyl5-(1-methylethyl)-3,4-heptadiene-2-one	9.22
6	31.5265	10.10620459	358	$C_{20}H_{22}O_6$	Columbin	22.4
7	32.2059	16.58073934	446	$C_{24}H_{30}O_8$	[1*S*-(1α,3aα,4α,6aα)]-1*H*,3*H*-Furo-[3,4-*c*]furan tetrahydro phenyl	94.8
8	32.9719	4.725871145	376	$C_{20}H_{32}O_5$	[1*S*(1α,4aα,4bβ,8β,8aα,10aβ)]-1-Phenanthrene carboxylic acid tetradecahydro-7-[2-methoxy-2-oxothylidene]-1,4a,8-timethyl-9-oxo-methylester	10.7
9	34.3775	7.338484499	349	$C_{19}H_{27}NO_5$	2,6-Ditetra butyl-4-methoxy phenylester-1nitro cyclopropane carboxylic acid	13.1
10	36.5823	5.730121921	220	$C_{13}H_{16}O_3$	3,4-Dihydro-4,4,5,8-tetramethyl-coumarin-6-ol	4.96
11	43.9829	5.97908391	416	$C_{25}H_{36}O_5$	Acetic acid 17-acetoxy-4,4,10,13-tetramethyl-7-oxo-2,3,4,7,89.0.11.12. 13.14.15.16.17-tetradecahydro-1*H*-cyclopenta(a)-phenanthren-3yl-ester	12.2
12	44.6091	5.535500245	376	$C_{20}H_{32}O_5$	[1*S*(1α,4aα,4bβ,8β,8aα,10aβ)]-1-phenanthrene carboxylic acid tetradecahydro-7-[2-methoxy-2-oxothylidene]-1,4a,8-timethyl-9-oxo-methylester	10.7

[a]Name of the compounds and percentage of probability was collected from compound library prepared during GC-MS analysis

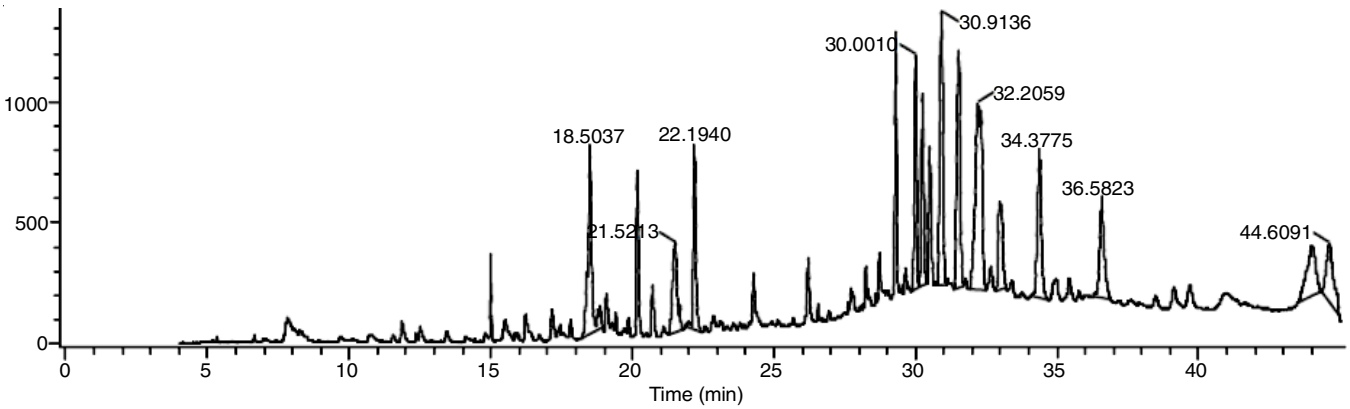


Fig. 1. GC-MS chromatogram for chloroform extract of *Tinospora cardifolia*

compounds were identified in chloroform extract of *Tinospora cardifolia*. The structure of compounds was confirmed by the NIST search library prepared during analysis. Out of all the compounds reported molecule 6 (10.10 %) and molecule 7 (16.58 %) were found in higher concentrations at the retention time of 31.52 and 32.20 min, respectively.

UV and FTIR analysis: The UV sstudy of the extract was carried out using phosphate buffer (pH 6.8) at 30 µg/mL. The spectrum (Fig. 2) exhibited the absorbance maxima at 215 nm and showed the π-π transitions. The FTIR analysis was carried out on the extracts for the compounds found in GC-MS analysis. The FTIR peaks (Fig. 3) at 3391.14 (-OH, *str.*); 2919.80 (-OCH_3, *str.*); 1620.70 (C=O, *str.*); 1384.61(-C-O-, *str.*) and 1153.07(-C-N, *str.*) exhibited the presence of reported compounds in GC-MS analysis.

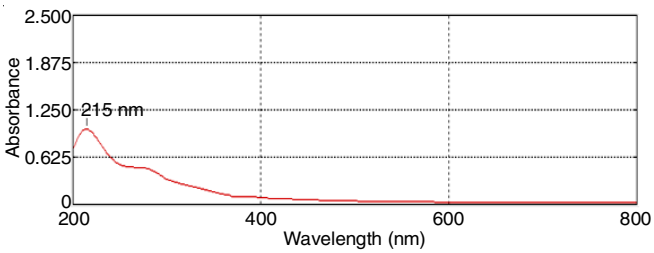


Fig. 2. UV-spectrum for chloroform extract of *Tinospora cardifolia*

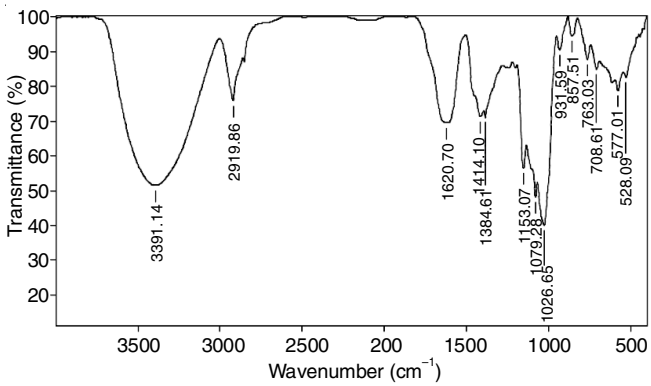
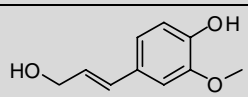
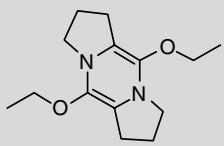
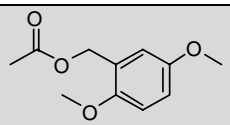
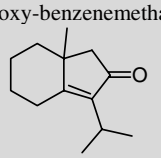
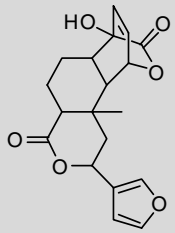
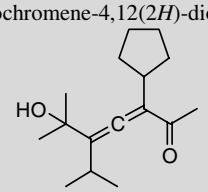
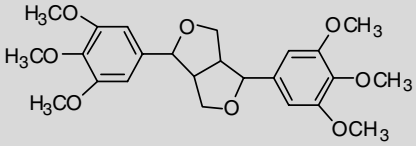
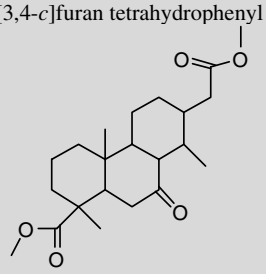
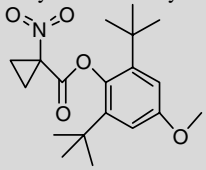
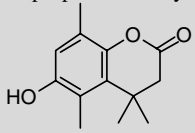


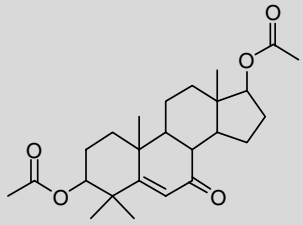
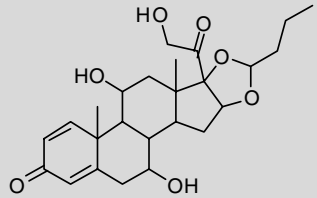
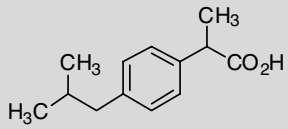
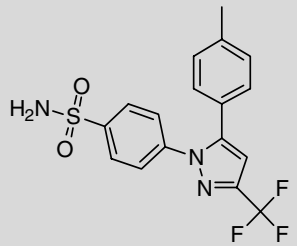
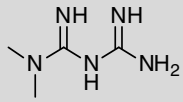
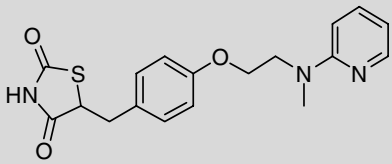
Fig. 3. IR spectra for chloroform extract of *Tinospora cardifolia*

Molecular property: The shape index, molecular flexibility and molecular complexity play a vital role in drug action and binding with the receptor molecules. Generally, linear shape molecules are considered ideal drug molecules [35]. Whereas molecules with high flexibility and low molecular complexity are considered for proper binding affinity toward the receptors [36,37]. The molecular property of the molecules was determined by Orasis data warrior software. The results shown that molecules-1, 2 and 3 are linear in shape whereas molecules-6 to 12 are spherical (Table-3). Except for molecule-9, all the molecules showed low molecular flexibility. Similarly, all the

TABLE-3
MOLECULAR PROPERTIES ALONG WITH SMILE NOTATION FOR THE COMPOUNDS IDENTIFIED BY SPECTRAL ANALYSIS FOR CHLOROFORM EXTRACT OF *Tinospora cardifolia*

Molecules	Structure and name of compounds	Smile notation	Shape index[a]	Molecular flexibility[b]	Molecular complexity[c]
1	 4-[(1*E*)-3-hydroxy-1-propenyl]-2-methoxy phenol	Oc1ccc(cc1OC)\C=C\CO	0.69231	0.39309	0.66172
2	 5,10-Diethoxy-2,3,7,8-tetra hydro-1*H*,6*H*-dipyrrolo [1,2a,1',2'd]pyrazine	CCOC2=C3CCCN3C (OCC)=C1CCCN12	0.55556	0.29736	0.88256

3	 2,5-Dimethoxy-benzenemethanol acetate	COc1ccc(cc1COC(C)=O)OC	0.6	0.44821	0.65645
4	 1*H*-2-Indenone,2,4,5,6,7,7a-hexahydro-3-(1-methylethyl)-7a-methyl	CC(C)C=1C(=O)CC2 (C)CCCCC=12	0.5	0.35408	0.77373
5	 Columbin/(10*S*)-2-(furan-3-yl) -7-hydroxy-10b-methyl-4a,5,6,6a,7,10,10a,10b-octahydro-1*H*-10,7-(epoxymethano)benzo[f] isochromene-4,12(2*H*)-dione	O=C4OC(CC3(C)C4CCC1C3 C2C=CC1(O)C(=O)O2)c5ccoc5	0.44	0.37897	0.96293
6	 3-Cyclopentyl-6-hydroxy-6-methyl5-(1-methylethyl)-3,4-heptadiene-2-one	O=C(C)\C(=C=C(/C(C)C) C(C)(C)O)C1CCCC1	0.44444	0.44235	0.63962
7	 [1*S*-(1α,3aα,4α,6aα)]-1*H*,3*H*-Furo-[3,4-*c*]furan tetrahydrophenyl	COc1cc(cc(OC)c1OC)C4OCC3C4 COC3c2cc(OC)c(OC)c(OC)c2	0.5	0.31125	0.88837
8	 [1*S*(1α,4aα,4bβ,8β,8aα,10aβ)]-1-Phenanthrene carboxylic acid tetradecahydro-7-[2-methoxy-2-oxothylidene]-1,4a,8-timethyl-9-oxo-methylester	O=C(OC)CC1CCC2C(C1C)C(=O) CC3C(C)(CCCC23C)C(=O)OC	0.51852	0.51063	0.88017
9	 2,6-Di-*tert*-butyl-4-methoxyphenyl-1-nitro-cyclopropane-carboxylate	O=N(=O)C1(CC1)C(=O)Oc2c (cc(OC)cc2C(C)(C)C)C(C)(C)C	0.44	0.55834	0.7679
10	 3,4-Dihydro-4,4,5,8-tetramethyl-coumarin-6-ol	Cc2c(O)cc(C)c1OC(=O)CC(C)(C)c12	0.5	0.2281	0.83059

11	 4,4,10,13-Tetramethyl-7-oxo-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]-phenanthrene-3,17-diyl diacetate	CC(=O)OC4CCC3(C)C2CCC1(C)C(CCC1OC(C)=O)C2C(=O)C=C3C4(C)C	0.5	0.38054	0.94515
12	 1,7-Dihydroxy-8b-(2-hydroxy acetyl)-6a,8a-dimethyl-10-propyl-6a,6b,7,8, 8a,8b,11a,12, 12a,12b-decahydro-1*H*-naphtho-[2',1':4,5]indeno[1,2-*d*][1,3]dioxol-4(2*H*)-one	OCC(=O)C53OC(OC5CC2C1C(O)CC4=CC(=O)C=CC4(C)C1C(O)CC23C)CCC	0.46875	0.35148	1.0499
Ibuprofen		–	0.67	0.62	0.56
Celicoxib		–	0.5	0.47398	0.82757
Metformin		–	0.66667	0.79706	0.54931
Rosiglitazone		–	0.68	0.53192	0.70181

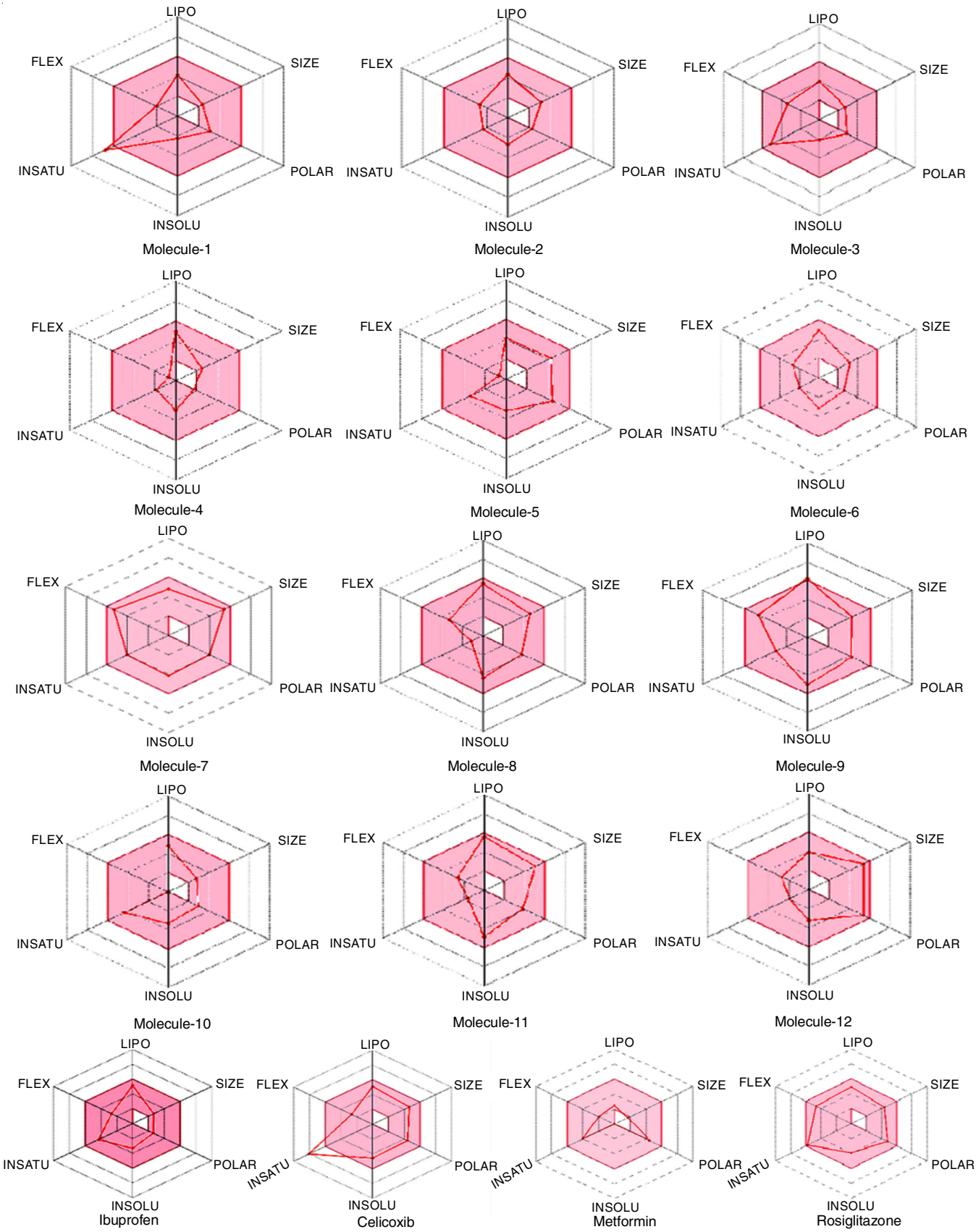
[a]Molecular shape index (spherical ≤ 0.5 ≤ linear); [b]Molecular flexibility (low ≤ 0.5 ≤ high); [c]Molecular complexity (low ≤ 0.5 ≤ high).

molecules shown higher molecular complexity compared to standards.

Physico-chemical property: The physico-chemical properties like molecular weight, partition coefficient, solubility, H-acceptors, H-donors [38], total surface area, relative polar surface area, TPSA (Å^2) [39], percentage of absorption, Fraction Csp3, molar refractive index [40] have great significance on biological activity and drug likeliness property of the molecules. The physico-chemical properties were calculated using Osiris data warrior and Csp3, while the molar refractivity was calculate using the Swiss ADME tool. All the molecules exhibited good drug likeliness characteristics with respect to the standard (Table-4). Based on the molecular and physico-chemical properties, the bioactivity reader is shown in Fig. 4.

Drug likeliness: The total drug likeliness characteristics like Drug likeness score, Lipinski, Ghose, Veber, Egan and Muegge rule. The bioavailability score of all the molecules was also calculated by using the Swiss ADME tool. All the molecules followed drug likeliness as per Lipinski, Ghose, Veber, Egan's rule (Table-5). Except for molecules 1, 4 and 9, all molecules followed Muegge rules. The bioavailability score was found to be 0.55 in respect of all the compounds. Out of 12 molecules found in GC-MS analysis, molecules 2, 5 and 12 exhibited positive drug likeliness values 0.54, 0.73 and 2.74, respectively. The results showed the good drug likeliness characteristics in comparison to standards.

Bioactivity score: The bioactivity score was determined on GPCR ligand (G-Protein coupled receptor), ion channel



The coloured zone is suitable physico-chemical space for oral bioavailability. LIPO (liphophilicity): 0.7 ≤ XLOGP3 ≤ +5.0; SIZE: 150 g/mol ≤ MV; POLAR (polarity): 20 Å^2 ≤ TPSA ≤ 130 Å^2; INSOLU (insolubility): 0 ≤ LOGS (ESOL) ≤ 5.0; INSATU (insaturation): 0.25 ≤ Fraction Csp^3 ≤ 1; FLEX (Flexibility): 0 ≤ Number of rotatable bond ≤ 9

Fig. 4. Bioactivity radar of the molecules identified in GC-MS analysis in comparison to standards

TABLE-4
PHYSICO-CHEMICAL PROPERTY OF THE MOLECULES IDENTIFIED IN GC-MS ANALYSIS

Molecules	m.w.	cLog P[a]	cLog S[b]	Solubility	H-Acceptors	H-Donors	Total surface area	Relative PSA[c]	TPSA[d] (Å²)	% abs[e]	Fraction Csp3	MR[f]
1	180.202	1.4078	-1.739	Soluble	3	2	148.48	0.2438	49.69	91.857	0.12	41.92
2	250.341	3.2204	-4.85	Soluble	4	0	194.04	0.13966	24.94	100.396	0.2	51.02
3	210.228	1.4069	-1.946	Soluble	4	0	171.55	0.25089	44.76	93.5578	0.67	70.14
4	192.301	3.2709	-2.945	Soluble	1	0	155.66	0.08377	17.07	103.111	0.5	77.84
5	344.362	0.6348	-3.103	Soluble	6	1	234.16	0.31299	85.97	79.3404	0.25	69.9
6	250.38	3.736	-2.932	Soluble	2	1	211.29	0.12372	37.3	96.1315	0.58	85.55
7	446.494	2.5818	-3.074	Soluble	8	0	337.1	0.23732	73.84	83.5252	0.29	68.86
8	378.507	3.4309	-4.004	Moderately soluble	5	0	287.19	0.20586	69.67	84.9639	0.89	121.79
9	349.425	3.6297	-4.747	Poorly soluble	6	0	265.75	0.2388	81.35	80.9343	0.73	71.11
10	220.267	2.4543	-3.284	Soluble	3	1	167.07	0.21632	46.53	92.9472	0.5	116.82
11	416.556	4.2141	-4.819	Moderately soluble	5	0	307.31	0.19238	69.67	84.9639	0.71	96.01
12	446.538	1.2467	-3.423	Soluble	7	3	309.83	0.27557	113.29	69.915	0.7	108.59
Ibuprofen	206.284	3	-2.89	Moderately soluble	2	1	172.9	0.15119	37.3	94.63	0.12	89.96
Celicoxib	381.377	2.5888	-4.174	Moderately soluble	5	1	259.56	0.23767	86.36	79.2058	0.12	89.96
Metformin	129.166	-1.7137	0.827	Very soluble	5	4	108.92	0.56445	88.99	78.29845	0.5	36.93
Rosiglitazone	357.433	2.1619	-3.666	Moderately soluble	6	1	269.07	0.29565	96.83	75.59365	0.28	101.63

[a]P=[n-Octanol]/[Water]; (cLog P); [b]S = Water solubility in mol/L at pH = 7.5 (25 °C) (cLog S); [c]Relative polar surface area (Relative PSA); [d]Toplogical polar surface area (TPSA); [e]Percentage of absorption (%Abs); [f]Molar refractive index.

TABLE-5
DRUG LIKENESS OF THE MOLECULES IDENTIFIED IN GC-MS ANALYSIS

Molecules	Drug likeness	Lipinski	Ghose	Veber	Egan	Muegge	Bioavailability score
1	-1.5442	Yes; 0 violation	Yes	Yes	Yes	No; 1 violation: MW < 200	0.55
2	0.5405	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
3	-1.5116	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
4	-4.7835	Yes; 0 violation	Yes	Yes	Yes	No; 2 violations: MW < 200, Heteroatoms < 2	0.55
5	0.7353	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
6	-3.4063	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
7	-0.886	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
8	-8.685	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
9	-7.9208	Yes; 0 violation	Yes	Yes	Yes	No; 1 violation: XLOGP3 > 5	0.55
10	-3.3599	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
11	-0.3236	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
12	2.7463	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55
Ibuprofen	0.085	Yes; 0 violation	No; 1 violation: WLOGP > 5.6	Yes	Yes	Yes	0.55
Celicoxib	-8.1085	Yes; 0 violation	No; 1 violation: WLOGP > 5.6	Yes	Yes	Yes	0.55
Metformin	3.5915	Yes; 0 violation	No; 3 violations: MW < 160, WLOGP < -0.4, MR < 40	Yes	Yes	No; 2 violations: MW < 200, #C < 5	0.55
Rosiglitazone	7.5038	Yes; 0 violation	Yes	Yes	Yes	Yes	0.55

modulator, a kinase inhibitor, nuclear receptor ligand, protease inhibitor and enzyme inhibitor. A bioactivity score of more than 0 is considered as a good, -0.50 to 0 consider as moderate and less than 0.5 is considered as inactive compounds [41]. Based on the results (Table-6), the bioactivity order for the molecules in respect to target receptors are Enzyme inhibitor > Nuclear receptor > GCPR ligand > Protase inhibitor > Ion channel modulator > Kinase inhibitor. Out of 12 molecules isolated molecules 4, 5, 6, 7, 8, 9, 10, 11 and 12 shown bioactivity score more than 0 in respect of different receptors.

Toxicity profiles: The toxicity potential of the molecules was determined for the mutagenic, tumorigenic, reproductive effects, irritant properties by Osiris data warrior. Table-7 shows that molecules 6 and 11 have a high and low reproductive effect,whereas molecules 5. 9 and 10 are irritant in nature.

TABLE-6
BIOACTIVITY SCORE OF THE MOLECULES IDENTIFIED IN GC-MS ANALYSIS

Molecules	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
1	-0.55	-0.05	-0.74	-0.30	-1.00	-0.08
2	-0.23	-0.33	-0.10	-0.21	-0.52	-0.01
3	-0.50	-0.20	-0.81	-0.48	-0.53	-0.19
4	-0.62	-0.14	-1.00	-0.23	-0.54	0
5	0.55	-0.17	-0.32	0.66	-0.13	0.47
6	-0.32	0.10	-0.44	0.12	-0.27	0.09
7	-0.03	-0.25	-0.19	-0.10	-0.16	0.01
8	0.14	0.12	-0.57	0.45	0.12	0.39
9	0.04	0.13	-0.10	0.11	0.05	0.10
10	-0.29	-0.29	-0.69	0.04	-0.78	-0.06
11	0.05	-0.04	-0.62	0.60	0.01	0.63
12	0.20	-0.35	-0.64	1.26	0.25	0.65
Ibuprofen	-0.17	-0.01	-0.72	0.05	-0.21	0.12
Celicoxib	-0.06	-0.27	0.01	-0.28	-0.06	0.17
Metformin	-1.44	-0.82	-2.47	-3.48	-1.11	-1.59
Rosiglitazone	0.15	-0.65	-0.61	0.35	-0.21	-0.07

TABLE-7
TOXICITY PROFILES OF THE MOLECULES IDENTIFIED IN GC-MS ANALYSIS

Molecules	Mutagenic	Tumorigenic	Reproductive effective	Irritant
1	None	None	None	High
2	None	None	None	None
3	None	None	None	None
4	None	None	None	None
5	None	None	None	High
6	None	None	High	None
7	None	None	None	None
8	None	None	None	None
9	None	None	None	High
10	None	None	None	High
11	None	None	Low	None
12	None	None	None	None
Ibuprofen	None	None	None	None
Celicoxib	None	None	None	None
Metformin	High	None	High	None
Rosiglitazone	None	None	None	None

Pharmacokinetics profiles: Most of the biomolecules are absorbed by the active or passive diffusion process. GI-absorptivity is an important parameter for oral absorption of bimolecular substances. The small intestine has the largest area for absorption of drugs in the GI tract than the stomach due to and the permeability of its membrane [42]. As the intestine is considered a major site of absorption so prediction of human intestinal absorption (HIA) of drug compounds is necessary [43]. The blood-brain barrier controls the entry of drug molecules into the brain. The molecules which have drug likeliness properties may cross the blood-brain barrier and may cause some toxic effects. So it is important to predict the BBB penetrability as well as toxicity profile of the molecules [44].

The PGP played a vital role in the drug disposition process like urinary excretion mechanism, a biliary excretion mechanism. It is also an important factor absorption barrier of oral bioavailability and the blood-brain barrier, which limits the accumulation of drugs in the brain. The PGP inhibition causes drug interactions and also increases the accumulation of drugs in the brain [45]. Cytochrome P450 is the class of enzymes essential for the metabolism of drugs. Inhibition of cytochrome P450 (CYP) enzymes by a drug may decrease the metabolism of drugs and other metabolic processes [46]. The skin permeability of the drug substances is an important criterion for tropical applications. The measurement of the rate at which a molecule can cross the lipid bilayer membrane of the skin is called skin permeation coefficient (KP). It is expressed in cm/s and equal to diffusion coefficient by the width of the membrane [47].

The pharmacokinetic profile like GI absorption, BBB permeant, P-GP substrate, CYP inhibitory effect (1A2; 2C19; 2C9; 2D6, 3A4), Log Kp (skin permeation) was determined by the Swiss ADME tool. Fig. 5 shows that all the molecules have GI-absorption capacity, The molecules 1, 2, 3, 4, 6, 7, 8, 10 & 11 have blood-brain barrier penetrability. Similarly for molecules 5, 9 and 12 human intestinal absorptions (HIA) capacity is more. The molecules 5, 6 and 12 exhibited the PGP initiator effect whereas molecules 1.2. 3.4.7.8.9.10 and 11 exhibited PGP inhibitory effect shown (Table-8). Except for molecules 1, 4, 5 and 6, all other molecules have a CYP-inhibitory effect against different CYP inhibitors. The results also reported skin permeability of the molecules in the acceptable range.

Docking analysis: Docking is an important tool for the prediction of the predominant binding mode(s) of a ligand with a protein of known three-dimensional structure. From ligand-based approaches, it was found most of the molecules present in chloroform extract are biologically active with good pharmacokinetics and therapeutic profile [48]. Based on the fact preliminary docking analysis was carried out on different targeted proteins like 5zcb, 5ycp, 4pyp for α-glucosidase, PPAR gamma ligand binding and human glucose transporter GLUT1 inhibition and 1eqg, 3ln1 for COX-1 and COX-2 inhibitory effects in respect of all the compounds and results were compared with standards metformin, rosiglitazone, ibuprofen and celecoxib.

The results in Table-9, represent FullFitness (kcal/mol) and binding energy ΔG (kcal/mol) of the molecules. Molecules 7 and 12 have good PPAR gamma ligand binding affinity,

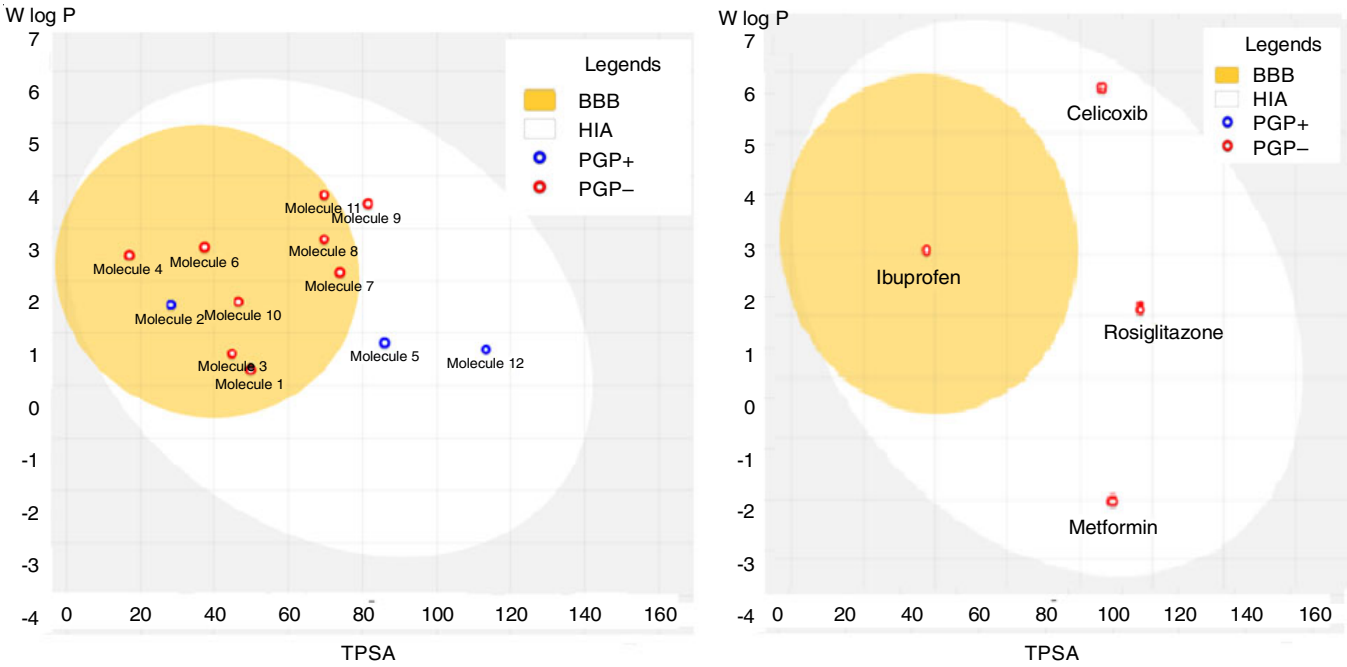


Fig. 5. Boiled egg diagram for the molecules identified in GC-MS analysis in comparison to standard

TABLE-8
PHARMACOKINETICS PROFILES OF THE MOLECULES IDENTIFIED IN GC-MS ANALYSIS

Molecules	GI absorption	BBB permeant	P-gp substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log Kp (skin permeation)
1	High	Yes	No	No	No	No	No	No	-6.13 cm/s
2	High	Yes	Yes	No	Yes	No	Yes	No	-6.33 cm/s
3	High	Yes	No	Yes	No	No	No	No	-6.48 cm/s
4	High	Yes	No	No	No	No	No	No	-5.19 cm/s
5	High	No	Yes	No	No	No	No	No	-7.15 cm/s
6	High	Yes	No	No	No	No	No	No	-5.63 cm/s
7	High	Yes	No	No	No	No	Yes	No	-6.98 cm/s
8	High	Yes	No	No	No	No	Yes	No	-5.73 cm/s
9	High	No	No	Yes	Yes	No	No	Yes	-4.65 cm/s
10	High	Yes	No	Yes	No	No	No	No	-5.59 cm/s
11	High	Yes	No	No	No	Yes	No	No	-5.77 cm/s
12	High	No	Yes	No	No	No	No	No	-8.04 cm/s
Ibuprofen	High	No	No	Yes	No	Yes	No	No	-6.21 cm/s
Celicoxib	High	No	No	Yes	No	Yes	No	No	-6.21 cm/s
Metformin	High	No	No	No	No	No	No	No	-7.84 cm/s
Rosiglitazone	High	No	No	No	Yes	Yes	Yes	Yes	-6.27 cm/s

whereas molecules 7 and 11 exhibits antidiabetic activity by GLUT-1 inhibition. To anti-inflammatory activity molecules, 7 and 9 have COX1 and 7 and 8 have COX2 inhibition. The binding pose and binding residue of the molecules with good binding affinity represents in Figs. 6-9. From the detailed analysis, it was found that among all the studied compounds, molecule-7 has a good inhibitory action on different targeted proteins.

Conclusion

The compounds reported in GC-MS analysis showed that [1S-(1α,3aα,4α,6aα)]-1*H*,3*H*-furo[3,4-*c*]furan tetrahydrophenyl (molecule 7) was found in the highest concentration in chloroform extract of plant *Tinospora cardifolia*. From the detailed analysis it was found that all the compounds exhibit good drug likeliness properties along with good toxicity potential and pharmacokinetic profiles. But due to the PGP and CYP inhibitory effect, some development at the formulation level is required on a pharmaceutical point of view. In docking analysis it was found that [1*S*-(1α,3aα, 4α,6aα)]-1*H*,3*H*-furo-[3,4-*c*]furan tetrahydrophenyl (molecule-7) have strong binding affinity on targeted proteins under investigation.

ACKNOWLEDGEMENTS

The authors are grateful to Srikrupa Institute of Pharmaceutical Sciences, Siddipet, India for providing the necessary research facilities. The authors are also thankful to SAIF, IIT Bombay for conducting the GC-MS and FTIR analysis of the extract.

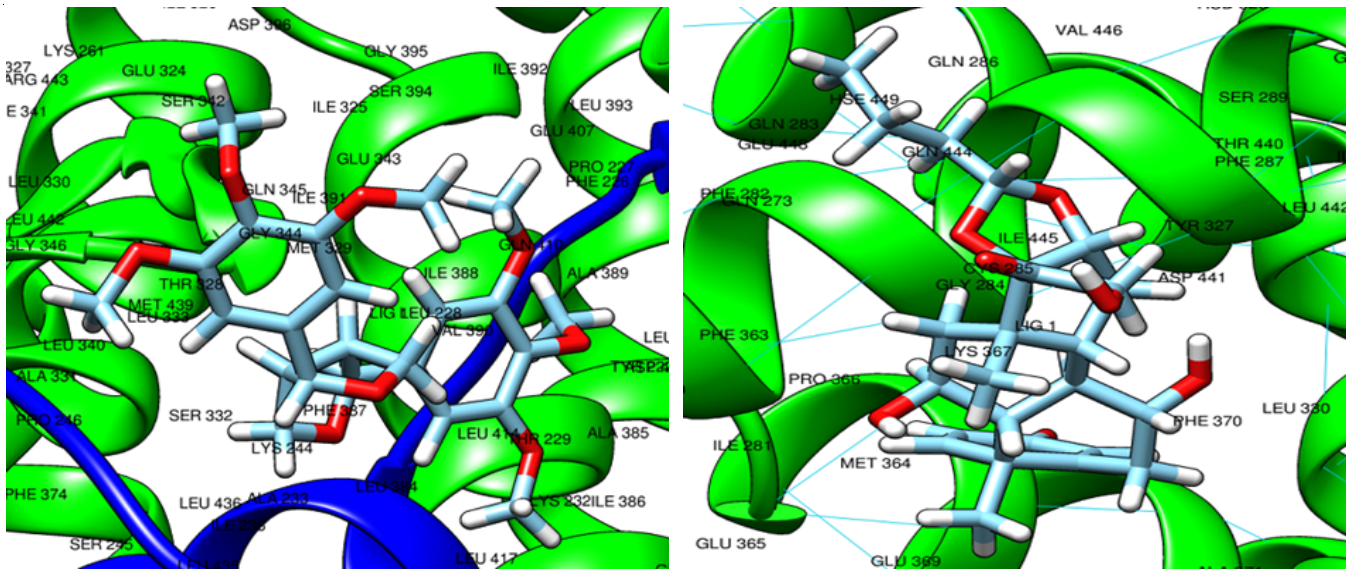


Fig. 6. Binding pose and amino acid residue for molecule 7 and 12 with 5ycp

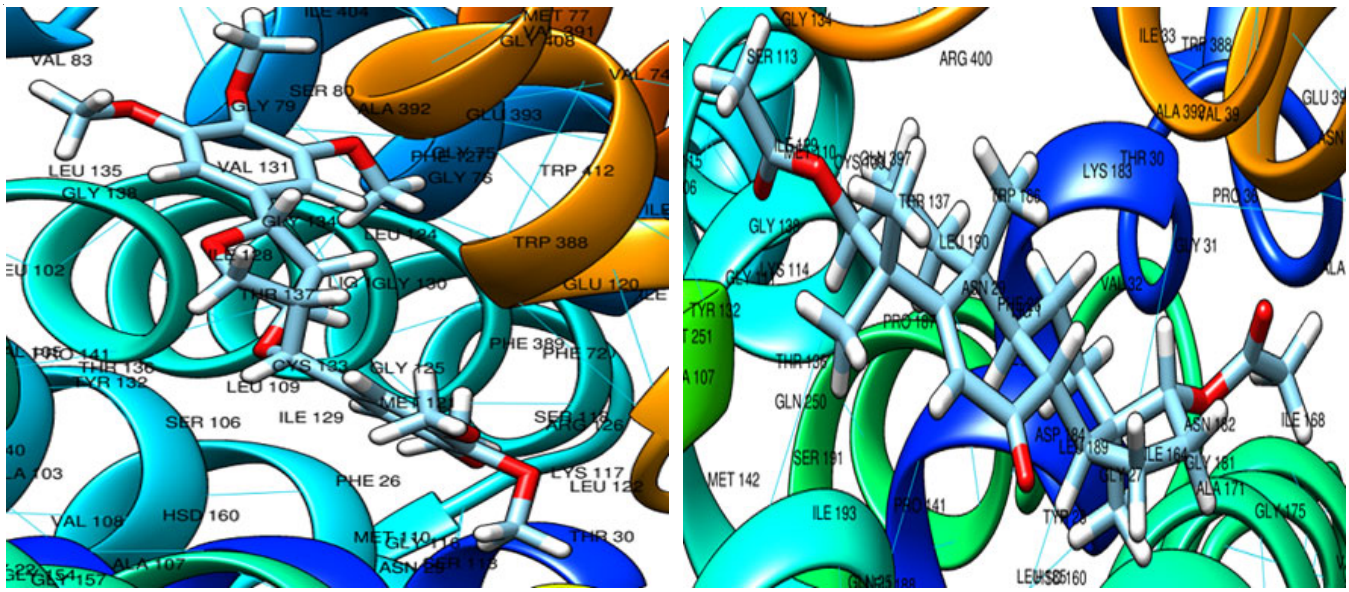


Fig. 7. Binding pose and amino acid residue for molecule 7 and 11 with 4pyp

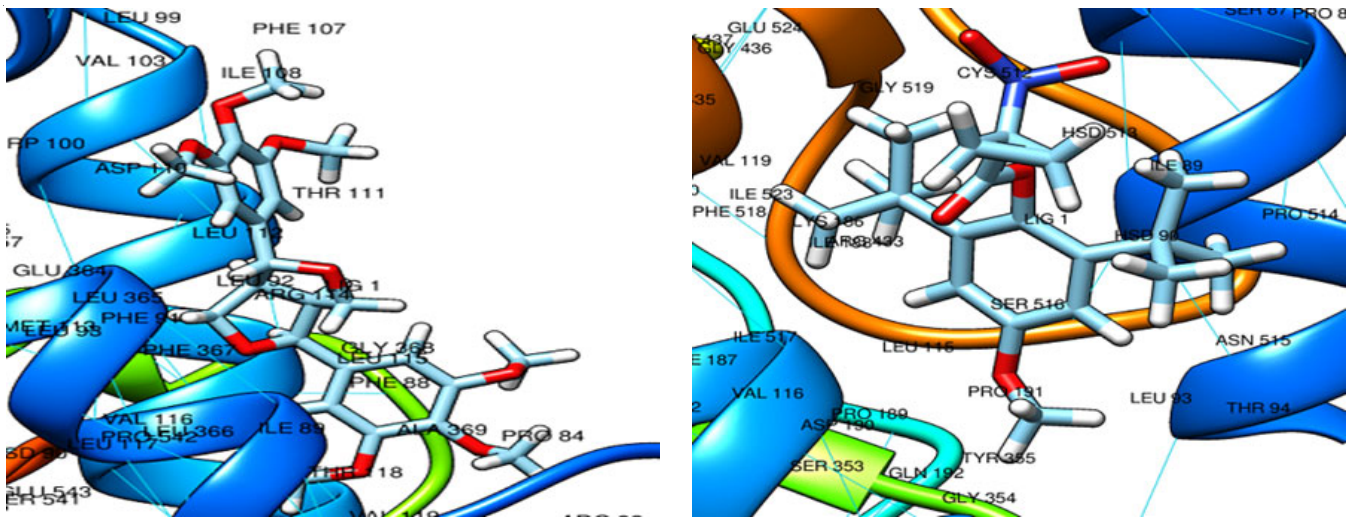


Fig. 8. Binding pose and aminoacid residue for molecule 7 and 9 with 1eqg

TABLE-9
BINDING SCORES OF THE MOLECULES IDENTIFIED IN GC-MS ANALYSIS

Molecules	5ycp		4pyp		1eqg		3ln1	
	Full fitness (kcal/mol)	Binding energy, ΔG (kcal/mol)	Full fitness (kcal/mol)	Binding energy, ΔG (kcal/mol)	Full fitness (kcal/mol)	Binding energy, ΔG (kcal/mol)	Full fitness (kcal/mol)	Binding energy, ΔG (kcal/mol)
1	-1798.00	-7.00	-1205.83	-6.82	-2137.25	-7.08	-2279.32	-7.24
2	-1781.24	-7.49	-1186.37	-7.41	-2122.96	-6.92	-2254.95	-7.19
3	-1809.76	-7.2	-1213.21	-6.85	-2152.11	-7.38	-2284.82	-7.53
4	-1802.61	-6.98	-1210.61	-6.90	-2146.55	-6.63	-2279.80	-6.44
5	-1735.37	-7.63	-1142.65	7.51	-2073.64	-7.32	-2211.01	-7.20
6	-1735.91	-7.47	-1140.11	-7.80	-2073.12	-6.92	-2209.41	-7.17
7	-1711.87	-8.84	-1132.40	-9.25	-2055.84	-8.24	-2184.25	-8.34
8	-1790.69	-8.22	-1186.07	-8.14	-2124.00	-7.33	-2270.88	-7.89
9	-1551.67	-7.93	-954.12	-8.21	-1886.43	-7.57	-2022.52	-7.37
10	-1790.48	-6.86	-1199.46	-7.04	-2126.07	-7.14	-2266.47	-6.72
11	-1775.32	-7.89	-1189.75	-8.44	-2102.62	-7.38	-2259.13	-7.82
12	-1719.07	-8.51	-1115.59	-7.90	-2047.18	-7.55	-2189.75	-7.57
Metformin	–	–	-1389.93	-6.38	–	–	–	–
Rosiglitazone	-1849.54	-8.50		–	–	–	–	–
Ibuprofen	–	–	–	–	-2158.39	-7.74	–	–
Celicoxib	–	–	–	–	–	–	-2275.95	-10.30

Binding energies -6.0 to -7.0 consider as less binding affinity, -7.0 to -8.00 consider as moderate binding affinity and -8.00 to -10.00 consider as good binding affinity.

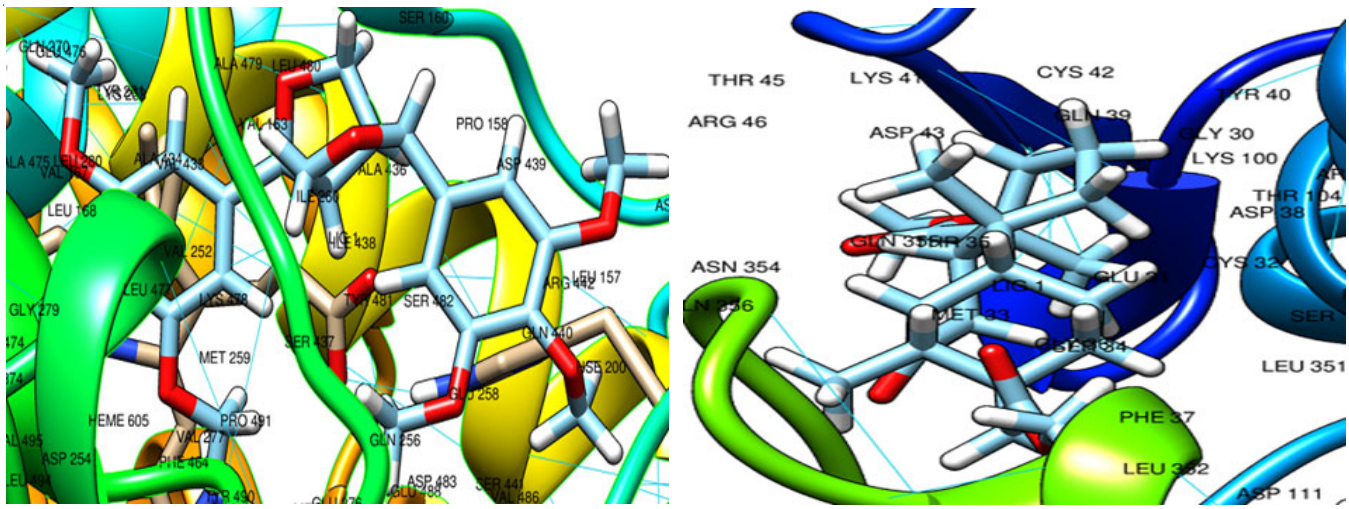


Fig. 9. Binding pose and amino acid residue for molecule 7 and 8 with 3ln1

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. A.S. Olabiyi, F.E. Nkemehule, O.A. Odukoya, T.A. Samuel and S.O. Ogbonnia, *Biochem. Pharmacol.*, **2**, 181 (2013).
2. M. Ekor, *Front. Pharmacol.*, **4**, 177 (2013); https://doi.org/10.3389/fphar.2013.00177
3. R. Wilken, M.S. Veena, M.B. Wang and E.S. Srivatsan, *Mol. Cancer*, **10**, 12 (2011); https://doi.org/10.1186/1476-4598-10-12
4. P. Pinal, P. Dharmik and P. Natvarlal, *Chin. J. Nat. Med.*, **10**, 269 (2012); https://doi.org/10.3724/SP.J.1009.2012.00269
5. D. Singh and P.K. Chaudhuri, *Nat. Prod. Commun.*, **12**, 299 (2017).
6. S. Saha and S. Ghosh, *Anc. Sci. Life*, **31**, 151 (2012); https://doi.org/10.4103/0257-7941.107344
7. P. Sharma, B.P.Dwivedee, D. Bisht, A.K. Dash and D. Kumar, *Heliyon*, **5**, e02437 (2019); https://doi.org/10.1016/j.heliyon.2019.e02437
8. Y.R. Chadha, The Wealth of India, Publication and Information Directorate, CSIR, New Delhi, pp. 251 (1976).
9. N.N. Rege, U.M. Thatte and S.A. Dahanukar, *Phytother. Res.*, **13**, 275 (1999); https://doi.org/10.1002/(SICI)1099-1573(199906)13:4<275::AID-PTR510>3.0.CO;2-S
10. A.K. Upadhyay, K. Kumar, A. Kumar and H.S. Mishra, *Int. J. Ayurveda Res.*, **1**, 112 (2010); https://doi.org/10.4103/0974-7788.64405
11. S. Ghosal and R.A. Vishwakarma, *J. Nat. Prod.*, **60**, 839 (1997); https://doi.org/10.1021/np970169z
12. P.A. Bafna and R. Balaraman, *Phytomedicine*, **12**, 264 (2005); https://doi.org/10.1016/j.phymed.2003.12.009
13. C.K. Atal, M.L. Sharma, A. Kaul and A. Khajuria, *J. Ethnopharmacol.*, **18**, 133 (1986); https://doi.org/10.1016/0378-8741(86)90025-5

14. S. Vedavathy and K.N. Rao, *J. Ethnopharmacol.*, **33**, 193 (1991); https://doi.org/10.1016/0378-8741(91)90178-G
15. G.C. Jagetia and S.K. Rao, *Evid. Based Complement Alternat. Med.*, **3**, 267 (2006); https://doi.org/10.1093/ecam/nel011
16. G.C. Jagetia and S.K. Rao, *Biol. Pharm. Bull.*, **29**, 460 (2006); https://doi.org/10.1248/bpb.29.460
17. S.S. Gupta, S.C.L. Verma, V.P. Garg and R. Mahesh, *Indian J. Med. Res.*, **55**, 733 (1967).
18. N. Wadood, A. Wadood and S. Shah, *Planta Med.*, **58**, 131 (1992); https://doi.org/10.1055/s-2006-961414
19. V. Sharma, R. Gupta and S. Shatruhan, *Int. J. Res. Pharm. Sci.*, **2**, 585 (2011).
20. V. Sharma, S. Sharma and R. Gupta, *J. Nat. Pharm.*, **2**, 125 (2011); https://doi.org/10.4103/2229-5119.86258
21. P. Stanely Mainzen Prince and V.P. Menon, *J. Ethnopharmacol.*, **65**, 277 (1999); https://doi.org/10.1016/S0378-8741(98)00164-0
22. P.S.M. Prince, V.P. Menon and G. Gunasekaran, *J. Ethnopharmacol.*, **64**, 53 (1998); https://doi.org/10.1016/S0378-8741(98)00106-8
23. P. Stanely, M. Prince and V.P. Menon, *J. Ethnopharmacol.*, **70**, 9 (2000); https://doi.org/10.1016/S0378-8741(99)00136-1
24. D. Jain, R.K. Dhuria, T. Sharma and T. Bothra, *Int. J. Chem. Stud.*, **6**, 2336 (2018).
25. S. Thillaivanan and K. Samraj, *Int. J. Herb. Med.*, **2**, 21 (2014).
26. G.I. Makrynitsa, M. Lykouras, G.A. Spyroulias and M.-T. Matsoukas, *In silico* Drug Design, John Wiley & Sons, Ltd: Chichester (2018).
27. A. Dhanik and L.E. Kavraki, Protein–Ligand Interactions: Computational Docking, John Wiley & Sons, Ltd., vol. 1 (2006).
28. D. Stumpfe and J. Bajorath, WIREs *Comput. Mol. Sci.*, **1**, 260 (2011); https://doi.org/10.1002/wcms.23
29. G.R. Marshall, C.D. Barry, H.E. Bosshard, R.A. Dammkoehler and D.A. Dunn, *Computer-Assisted Drug Des.*, **112**, 205 (1979); https://doi.org/10.1021/bk-1979-0112.ch009
30. B.J. McConkey, V. Sobolev and M. Edelman, *Curr. Sci.*, **83**, 845 (2002).
31. X.Y. Meng, H.X. Zhang, M. Mezei and M. Cui, *Curr. Computeraided Drug Des.*, **7**, 146 (2011); https://doi.org/10.2174/157340911795677602
32. A. Kumar, A. Voet and K. Zhang, *Curr. Med. Chem.*, **19**, 5128 (2012); https://doi.org/10.2174/092986712803530467
33. N. Chaudhary, M.B. Siddiqui and S. Khatoon, *J. Res. Indian Med.*, **13**, 543 (2014).
34. C.K. Kokate, Pharmacognosy, Nirali Prakashan: Pune, India, Ed.: 17, p. 106 (2001).
35. A. Nicholls, G.B. McGaughey, R.P. Sheridan, A.C. Good, G. Warren, M. Mathieu, S.W. Muchmore, S.P. Brown, J.A. Grant, J.A. Haigh, N. Nevins, A.N. Jain and B. Kelley, *J. Med. Chem.*, **53**, 3862 (2010); https://doi.org/10.1021/jm900818s
36. R. Rohs, I. Bloch, H. Sklenar and Z. Shakked, *Nucleic Acids Res.*, **33**, 7048 (2005); https://doi.org/10.1093/nar/gki1008
37. O. Méndez-Lucio and J.L. Medina-Franco, *Drug Discov. Today*, **22**, 120 (2017); https://doi.org/10.1016/j.drudis.2016.08.009
38. L.Z. Benet, C.M. Hosey, O. Ursu and T.I. Oprea, *Adv. Drug Deliv. Rev.*, **101**, 89 (2016); https://doi.org/10.1016/j.addr.2016.05.007
39. S. Prasanna and R.J. Doerksen, *Curr. Med. Chem.*, **16**, 21 (2009); https://doi.org/10.2174/092986709787002817
40. D. Ji, M. Xu, C.C. Udenigwe and D. Agyei, *Curr. Res. Food Sci.*, **3**, 41 (2020); https://doi.org/10.1016/j.crfs.2020.03.001
41. A. Husain, A. Ahmad, S.A. Khan, M. Asif, R. Bhutani and F.A. Al-Abbasi, *Saudi Pharm. J.*, **24**, 104 (2016); https://doi.org/10.1016/j.jsps.2015.02.008
42. W.S. Nimmo, *Clin. Pharmacokinet.*, **1**, 189 (1976); https://doi.org/10.2165/00003088-197601030-00002
43. M.D. Wessel, P.C. Jurs, J.W. Tolan and S.M. Muskal, *J. Chem. Inf. Comput. Sci.*, **38**, 726 (1998); https://doi.org/10.1021/ci980029a
44. W.M. Pardridge, *J. Cereb. Blood Flow Metab.*, **32**, 1959 (2012); https://doi.org/10.1038/jcbfm.2012.126
45. Y. Tanigawara, *Ther. Drug Monit.*, **22**, 137 (2000); https://doi.org/10.1097/00007691-200002000-00029
46. J.H. Lin and A.Y. Lu, *Clin. Pharmacokinet.*, **35**, 361 (1998); https://doi.org/10.2165/00003088-199835050-00003
47. R.O. Potts and R.H. Guy, *Pharm. Res.*, **9**, 663 (1992); https://doi.org/10.1023/A:1015810312465
48. G.M. Morris and M. Lim-WilbyA. Kukol, *Methods Mol. Biol.*, **443**, 365 (2008); https://doi.org/10.1007/978-1-59745-177-2_19