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Research Article

In Silico and *In Vitro* Study of R-Phenyl Alanine -S-Mandelate

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ABSTRACT
The reactive oxygen species (ROS) are generated during the metabolic food digestion activities. If they are not scavenged properly, they may damage the normal cells and give cancerous diseases. The small organic molecules are used to scavenge the ROS formed in the metabolic activities by undergoing redox reactions. Novel organic salt R-phenyl alanine -S-mandelate (RPASMA) was synthesised and was subjected to characterisation studies. The donor-acceptor interactions, charge transfer mechanism, hydrogen bonding interactions and van der Waals forces of attraction were taking place in the crystallization resulting in the formation of low energy gap organic salt. The low energy gap title compound showed the high chemical reactivity and the low chemical stabilty, gave an idea to proceed with its *In Silico* study and *In Vitro* study. The pharmacokinetic study, admet study and molecular docking study were performed for the title compound. The electrochemical activity and antioxidant activity of the title compound showed encouraging results. The anticancer activity was carried for the title compound using Liver carcinoma HepG2 cell-line and normal (Vero) cell line for the cell viabilty and cytotoxicity. The suitability of the title compound for the eradication of ROS was confirmed from the *In Silico* and *In Vitro* studies which may minimise the chances of deadly cancerous disease formation.

Keywords: ROS, RPASMA, In Silico, In Vitro, HepG2 cell-line, Vero cell-line.

INTRODUCTION

Reactive Oxygen Species (ROS) capable of causing damage to DNA, have been associated with carcinogenesis, coronary heart diseases and other health problems related to advancing age. ROS formed during metabolic activities undergo redox reactions[1]. ROS, continuously produced during normal physiological events, can easily initiate the oxidation of membrane lipids leading to the accumulation of lipid peroxides[2]. Synthetic new antioxidants like phenyl alanine mandelates can scavenge or prevent the generation of ROS which can protect the formation of free radicals and retard the process of many chronic diseases like cancer, neurodegenerative, inflammation and cardio vascular diseases. Phenolic compounds containing aromatic rings are undergoing electrochemical reactions[3]. The aromatic alpha hydroxy acid, mandelic acid (MA) readily undergoes electro oxidation. The essential aminoacid, L-phenyl alanine (LPA) donate lone pairs of electrons on the nitrogen for the hydrogen bonding interactions with MA resulting in the formation of low energy gap novel organic salts[4]. These salts undergo electrochemical reaction due to low chemical stabilty and high chemical reactivity, combine with the ROS generated during the metabolic activities and deactivate them. The synthesis and the characterisation studies of the title compound confirmed the formation of the novel organic salt[5]. The antioxidant activity of the title compound was compared with the electrochemical behaviour using cyclic voltammetry measurement and found the presence of antioxidant activity and antiradical power. The molecular docking and admet analysis were carried out to ensure the suitability of the title compound for anticancer activity. The vital internal organ of human beings Liver plays major role in the metabolic activity. The degenerative mechanism of liver cells help to detoxify xenobiotics[6]. The metabolic activity of food digestion is resulting in the ROS formation.The ROS formed may destroy the normal cells during the proliferation process. The liver carcinoma is a deadly disease which kills huge number of people in this busy world due to the lack of improper nutritious in take of on time food. The *In Vitro* study of the title compound to determine the cell viability and cytotoxicity in using liver carcinoma HepG2 cell-line and normal (Vero) cell-line has confirmed the ability of the title compound to act as a promising anticancer material.

experiment

Alfa Aesar 99% pure dl-mandelic acid (MA), Nice chemicals 98% pure L-phenyl alanine (LPA) were used for the synthesis of the title compound.

Synthesis of R - phenyl alanine -S- mandelate (RPASMA)

The novel organic salt of 1:1 MA and LPA was isolated as RPASMA by the hydrolysis of the organic salt bis-l-phenyl alanine mandelate (BLPAMA) using aqueous methanol. The mixture of BLPAMA and methanol were taken in the molar ratio 1:6 respectively in a clean beaker. The mixture was stirred well at room temperature and filtered. The clear pale yellow solution obtained was kept for slow

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evaporation at room temperature. The hydrolysis took place leading to the formation of RPASMA. Single crystals of the pale yellow coloured RPASMA were harvested after 20 days which showed homogenous on TLC and has the melting point of 174 ˚C. The yield obtained was 65%. Schematic representation of RPASMA synthes is

$C_6H_5CH(OH)COOH.2C_6H_5CH_2CH(NH_2)$ COOH. H_2O + Aqueous CH_3OH Bis-L-phenyl alanine mandelate (BLPAMA)

↓

$C_6H_5CH(OH)COOH.C_6H_5CH_2CH(NH_2)$ COOH
R-phenyl alanine -S-mandelate (RPASMA)

fourier transform infrared(ftir) spectral analysis

FTIR of the title compound in Fig.1 indicates –O-H stretching at 3296 cm^{-1}, -C-H stretch at 3086 cm^{-1}, CH_2 asymmetric stretch at 2866 cm^{-1}, carboxylic acid carbonyl group stretching at 1712 cm^{-1}, NH_2 deformation at 1598 cm^{-1}, carbon ring stretching at 1497 cm^{-1}, carboxylic acid in plane –O-H bending at 1437 cm^{-1}, carboxylate O=C-O$^-$ stretching at 1399 cm^{-1}, C-N stretching at 1190 cm^{-1}, 1099 cm^{-1}, CH-OH stretching at 1072 cm^{-1}, CH out of plane deformation at 869 cm^{-1}, 699 cm^{-1} respectively, C-C-CN bending at 584 cm^{-1}, C-C=O bending at 513 cm^{-1}, C-O-C bending at 425 cm^{-1}[7,8].

nmr analysis

Nuclear Magnetic Resonance (NMR) spectroscopy depends on the chemical environment of the compound namely electron density, field of magnet and the indirect dipole-dipole interaction leading to J-coupling. 500MHz FT-NMR was used to analyse the samples of phenyl alanine mandelates. Electron density rich nucleus shielded protons are observed in the upfield[9,10].

The proton NMR and the carbon NMR of the title compound in Fig.2. and Fig.3. were confirming the expected respective atoms present and the details were given in the Table - 1 and Table - 2 respectively.

biological activity

The title compound was subjected to antibacterial activity using both gram positive and gram negative bacteria by agar well diffusion method and the antifungal activity using Trichoderma species by agar well diffusion method. Both were found to be absent. The presence of antioxidant activity was confirmed by both DPPH scavenging activity and cyclic voltammetry method.

dpph free radical scavenging assay

The ability of the title compound to annihilate the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) was investigated by the method described by Blois 1958. Stock solution of the title compound was prepared to the concentration of 10 mg/ml. Different concentration of the extract (25, 50, 75, 100, 200 & 250 µg) of sample were added to an equal volume of methanolic solution of DPPH (0.1mM). The reaction mixture was incubated for 30 min at room temperature. The absorbance was recorded at 517 nm. Ascorbic acid was used as standard control. The annihilation activity of free radicals was calculated in % inhibition using the formula, % of Inhibition = (A of control – A of Test)/A of control* 100. The comparative DPPH scavenging activity, efficient concentration and radical power were shown in the Table - 3 and Table - 4 respectively.

cyclic voltammetry

The electrochemical oxidation of the title compound shows higher area under anodic wave form which corresponds to higher antioxidant capacity[11]. The presence of electron donating groups have lower half wave potential, higher antioxidant activity and higher reducing power. The increase in the separation of peak potentials indicates charge transfer mechanism in the title compound. The title compound posseses electron donating group, show lower half wave potential, enhanced antioxidant activity and high reducing power. The hydroxyl group and electron donating amino group in the title compound are resulting in the higher antioxidant activity. Radical scavenging activity, antiradical power due to structure property activity leads to the high antioxidant activity of the title compound and can be used as a fighting agent to nullify the ROS generated during metabolic activities[12] is shown in the Fig.4.

anti cancer property

The formation of ROS during metabolic activities, if they were not scavenged, may result in the formation of cancerous disease[13]. The process of scavenging ROS using small organic molecules which can undergo redox reaction may slow down the genesis of cancer and can act as anticancer compounds[14]. MTT assay using cancer cell line and normal (VERO) cell line for the organic synthetic compounds show the cell viability and cytotoxic effect of the compounds to act as anticancer material[15,16]. The synthetic organic compounds having high anti oxidant activity are prone to act as anticancer materials[17]. The increase in antioxidant power and anti radical power of the title compound, gave an idea to ensure the presence of the anticancer property of the title compound[18] and was subjected to cytotoxic activity against liver carcinoma cell line (HepG2) and Vero cell line (Normal non-cancerous cell line)[19].

mtt assay for cell viability using hep g2 and vero cell-lines

The MTT assay was based on the ability of live but not dead cells to reduce a yellow tetrazolium dye (MTT) to a purple formazan product. Cells were maintained in DMEM medium, supplemented with 10% Fetal Bovine Serum, at 37℃ in humidified atmosphere with 5% CO_2. The cells were plated in 96 well flat bottom tissue culture plates at a density of approximately 1.2 X 10^4 cells/well and allowed to attach overnight at 37℃. The medium was then discarded and cells were incubated with different concentrations of the samples (25, 50, 75,100& 125µg) for 24 hours. After the incubation, medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After 4 hours, the medium was discarded and 100µl of DMSO was added to dissolve the formazan crystals[20]. Then the absorbance has been read at 570 nm in a microtitre plate reader. Cyclophosphamide was used as a positive control. Cell survival was calculated by the following formula: Viability % = (Test OD/ Control OD) X 100; Cytotoxicity % = 100 – Viability %

Table 1: Proton NMR of RPASMA

Sample	RPASMA
Solvent	CD3-SO-CD3
Solvent peak	2.5-2.6
N-H Proton	2.94
CH2 Proton	3.10
CH Proton	3.70
C-OH Proton	5.00
Aromatic protons	6.5-8.0

Table 2: Carbon NMR of RPASMA

Sample	RPASMA
Solvent	CD3-SO-CD3
Solvent peak	39-40
C - H Carbon	37.0
C-N Carbon	-
C - OH Carbon	72.9
Aromatic carbons	120-140
Carbonyl carbon	174.6

Table 3: Comparison of DPPH scavenging activity

Concentration	% Inhibition	
(µg)	ASCORBIC ACID	RPASMA
25	58.88	40.55
50	64.42	53.83
100	83.09	63.28
250	96.44	74.77

Table 4: Efficient concentration and anti radical power

SAMPLE	IC 50 (µg)	ARP (µg)
RPASMA	151.15	0.00662

The cell viability, cytotoxicity and efficient concentration or half maximal inhibitory concentration of the title compound using HepG2 cell-line and normal (Vero) cell-line were shown in the Table - 5 and Table - 6 respectively. PC- Positive control (Cyclophosphamide)

docking study

Molecular docking is one of the most frequently used methods in structure-based drug design, due to its ability to predict the binding-conformation of small molecule ligands to the appropriate target binding site[21]. Characterisation of the binding behaviour plays an important role in rational design of drugs as well as to elucidate fundamental biochemical processes[22]. The associations between biologically relevant molecules such as proteins, nucleic acids, carbohydrates and lipids play a central role in signal transduction. Furthermore, the relative orientation of the two interacting partners may affect the type of signal produced (e.g., agonism vs antagonism). Therefore, docking is useful for predicting both the strength and type of signal produced[23]. ADT (Autodock Tools) which amongst the other tools helps to set up bonds that will treat as rotatable in the ligand[24]. This enables us to analyze the docking interaction between the drug (ligand) and the drug target (receptor) in-silico[25].

docking of c-kit receptor protein-tyrosine kinase

The Autodock tool has been used for studying the interaction among c-Kit receptor protein-tyrosine kinase and R-phenyl Alanine-S-mandelate (RPASMA) using Autodock software and observed that the sample was having anticancer property and the results were shown in Fig.5.

step 1 - preparing the protein

Protein Data Bank (PDB) files can have a variety of potential problems that needs to be corrected before they can be used in AutoDock. These potential problems include missing atoms, added waters, more than one molecule, chain breaks, alternate locations *etc.* The water molecules have to be removed and polar water molecules have to be added and save in "pdb" format. The receptor file used by AutoDock must be in "pdbqs" format which is pdb plus 'q' charge and 's' solvation parameters, AtVol, the atomic fragmental volume, and AtSolPar, the atomic solvation parameter, which are used to calculate the energy contributions of desolvation of the macromolecule by ligand binding.

Step 2 - preparing the ligand

Before docking the partial atomic charges are applied to each atom of the ligand. We also distinguish between aliphatic and aromatic carbons: names for aromatic carbons start with 'A' instead of 'C'. AutoDock ligands are written in files with special keywords recognized by AutoDock. The root is a rigid set of atoms, while the branches are rotatable groups of atoms connected to the rigid root. The TORSDOF for a ligand is the total number of possible torsions in the ligand minus the number of torsions that only rotate hydrogens. TORSDOF is used in calculating the change in free energy caused by the loss of torsional degrees of freedom upon binding. After all the above conditions are set, then the ligand is saved in "pdbq" format.

step 3 - preparing the docking parameter file

The docking parameter file tells AutoDock which map files to use, the ligand molecule to move, what its center and number of torsions are, where to start the ligand, which docking algorithm to use and how many runs to do. It usually has the file extension, "dpf".

RESULTS AND DISCUSSION

The molecule which serves as a ligand was first analysed for the Lipinskis rule. It has been found that the compound satisfied the criteria to act as a ligand and it interacted with the protein[26]. The energy of the protein for RPASMA was +0.62Kcal/mol. The energy for the ligand was +0.04 Kcal/mol. Docking is an insilico approach where the possibility of interaction between a target protein and a ligand is predicted. The non-bonded interactions between the ligand and the protein CDK5 along with the docking score serves as a measure of the feasibility for the complex formation[27]. The docking study of RPASMA supports the anticancer property.

admet study

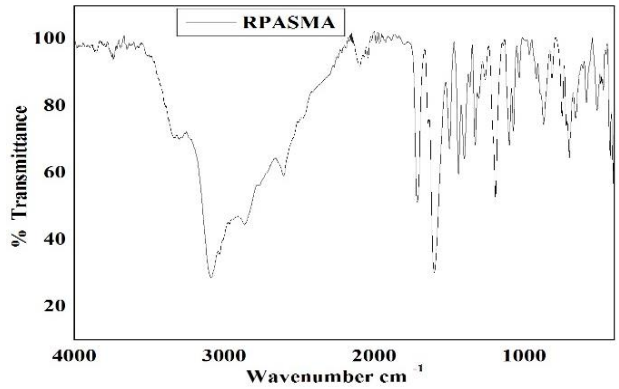


Figure 1: FTIR spectrum of RPASMA

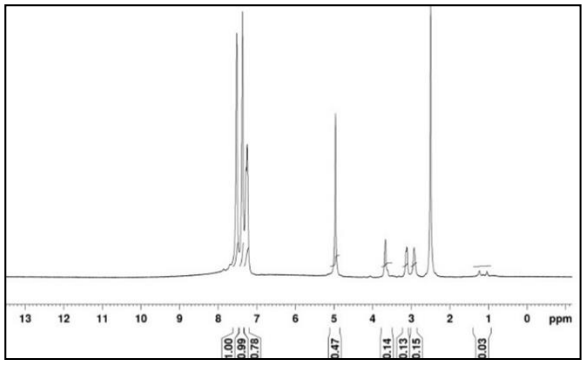


Figure 2: Proton NMR of RPASMA

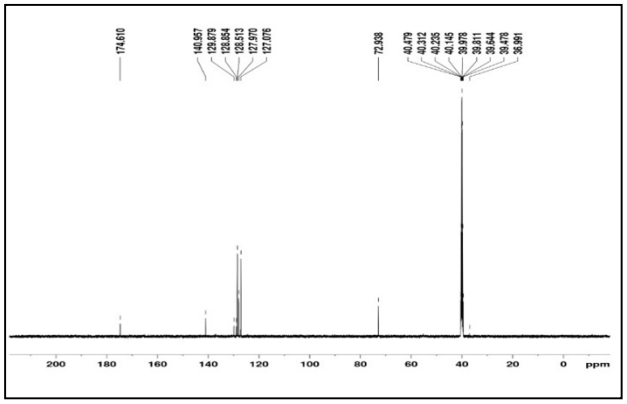


Figure 3: Carbon NMR of RPASMA

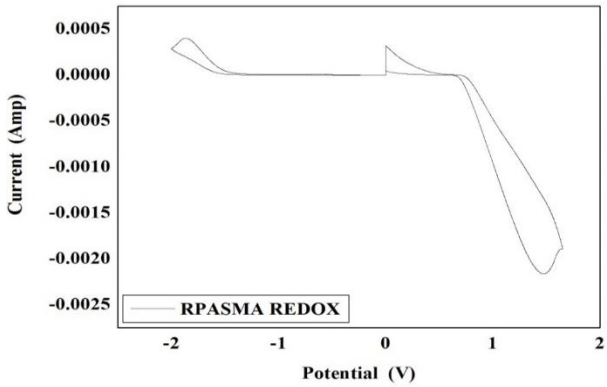
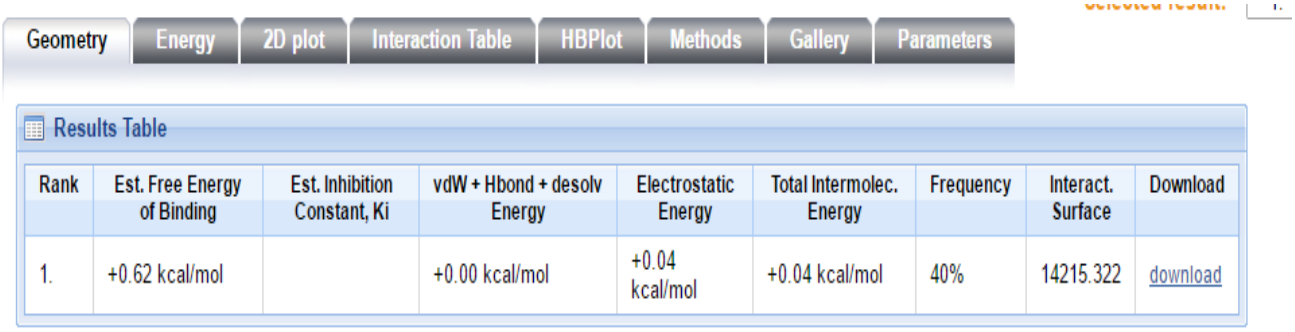


Figure 4: CV of RPASMA

Table 5: Percentage of cell viability and cytotoxicity of sample RPASMA against HepG-2 cell- line and Vero cell- line

Test	Sample - RPASMA (µg)					
	25	50	75	100	125	PC
% of Viability in HepG2 cell-line	65.69	63.2	58.65	56.47	52.08	44.81
% of Cytotoxicity in HepG2 cell-line	34.31	36.8	41.35	43.53	47.92	55.19
% of Viability in Vero cell-line	77.17	71.84	66.93	61.76	58.02	44.81
% of Cytotoxicity in Vero cell-line	22.83	28.16	33.07	38.24	41.98	55.19



Admet provides the latest and the most comprehensive manually curated data for diverse chemicals associated with known Absorption, Distribution, Metabolism, Excretion and Toxicity profiles. Admet is a user-friendly interface created to search for ADMET properties profiling by name, CASRN and similarity search[28]. In addition, admet can predict about 50 ADMET endpoints by our recent development chem-informatics-based toolbox, entitled ADMET-Simulator which integrates high quality and predictive QSAR models[29]. RPASMA sample was subjected to ADMET analysis and the results were shown in Table -7.

CONCLUSION

The characteristic studies, antioxidant activity, antiradical power, In Silico and in Vitro studies of the title compound

Table 6: Efficient concentration / half maximal inhibitory concentration of RPASMA using HepG2 cell-line and Vero cell-line

Sample	IC50 / HepG2 (µg)	IC50 / Vero (µg)
RPASMA	143.77	164.04

Table 7: ADMET parameters of RPASMA

Absorption	
Blood-Brain Barrier	BBB+
Human Intestinal Absorption	HIA+
Caco-2 Permeability	Caco2+
P-glycoprotein Substrate	Non-substrate
P-glycoprotein Inhibitor	Non-inhibitor Non-inhibitor
Renal Organic Cation Transporter	Non-inhibitor
Metabolism	
CYP450 2C9 Substrate	Non-substrate
CYP450 2D6 Substrate	Non-substrate
CYP450 3A4 Substrate	Non-substrate
CYP450 1A2 Inhibitor	Non-inhibitor
CYP450 2C9 Inhibitor	Non-inhibitor
CYP450 2D6 Inhibitor	Non-inhibitor
CYP450 2C19 Inhibitor	Non-inhibitor
CYP450 3A4 Inhibitor	Non-inhibitor
CYP Inhibitory Promiscuity	Low CYP Inhibitory Promiscuity
Toxicity	
Human Ether-a-go-go-Related Gene Inhibition	Weak inhibitor Non-inhibitor
AMES Toxicity	Non AMES toxic
Carcinogens	Non-carcinogens
Fish Toxicity	High FHMT
Tetrahymena Pyriformis Toxicity	High TPT
Honey Bee Toxicity	Low HBT
Biodegradation	Ready biodegradable
Acute Oral Toxicity	III
Carcinogenicity (Three-class)	Non-required

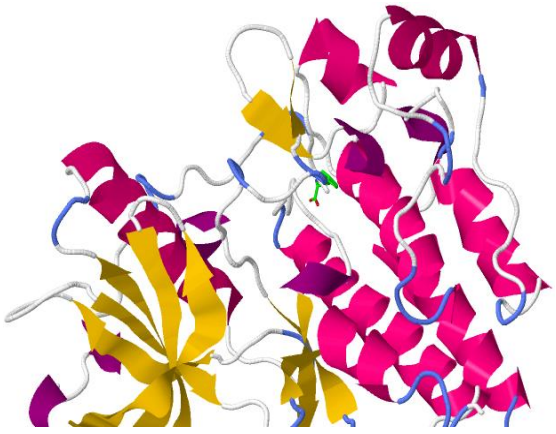


Figure 5: Auto Dock parameters of RPASMA

indicates the nullifying of metabolic ROS generated and the suitability of the title compound to use in the deadly cancerous disease treatment.

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