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Synthesis, Characterization and Anticancer Activity of (E) - N-ethyl-3 - (4- hydroxy- 3- methoxyphenyl) -N- methylacrylamide Analogue of Ferulic acid

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ABSTRACT

In our study to identify potent anti-inflammatory, antioxidant and antimicrobial (E)- N-ethyl-3 - (4- hydroxy- 3- methoxyphenyl) -N- methylacrylamide analogue have been design and synthesized from ferulic acid and the structure of the analogue determined by 1H NMR and C13 NMR of spectroscopic analysis. Docking studies with MDM2 protein a critical negative regulator of the tumor suppressor p53 and DNA docking studies also conduct with calf DNA which was carried out on Autodock programme. DNA binding studies were performed by UV–Visible spectroscopy. The synthesized compounds were tested for their anti-cancer activity by MTT assay method, antioxidant activity by DPPH and superoxide free radical methods. Antibacterial activity against pseudomonas aeruginosa and staphylococcus aureus by disk diffusion method. According to these results it can be suggested that developed compound has exhibit to significant anticancer activity, compared to parent compound of ferulic acid as standard.

Keywords: *Ferulic acid, p53-MDM2, DNA docking, UV Visible DNA binding study, Anticancer agents*

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INTRODUCTION

Ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid) is a phenolic acid isolated for the 1st time from Ferula foetida (Apiaceae) around the middle of the 19th century [1]. To overcome the side effects caused by regular approved drugs in clinical set up, natural plant derived compounds and its derivatives are used as an alternative strategy to target many diseases. Presently researchers are going on to explore the potential of ferulic acid in the treatment of cancer. Due to the biological importance of the amide moieties of FA we become interested in the synthesis of novel ferulic acid analogue that contain amide moiety [2]. Mouse double minute 2 (MDM2)expression is up-regulated in diverse cancers, resulting in a loss of p53-dependent activities, such as apoptosis and cell-cycle arrest [3], which leads to poor prognosis and treatment failure in present day chemotherapeutics to overcome this we concentrated on ferulic acid derivatives as potential chemotherapeutics for cancer. Present study we focused on to design, synthesis, characterization and biological activity of ferulic acid analogues among them we will publishing one of the analogue (E)- N-ethyl-3 - (4- hydroxy- 3- methoxyphenyl) -N- methylacrylamide (FAA4)). We also investigated the molecular interaction of analogues with MDM2 protein using docking study and DNA binding UV spectroscopic studies and FA analogue, biological functions were finally validated by biological activity studies such as antioxidant, antibacterial activities and anti cancer studies in cancer cell lines.

MATERIAL AND METHODS

Chemical Synthesis

All the analytical grade chemicals and solvents were purchased from Sigma-Aldrich and Merck. Ferulic acid acid was purchased from Bangalore (India). The reaction process and purity were monitored by using TLC Silica gel 60 F254 aluminium sheets (Merck, Darmstadt, Germany) developed in mobile phase containing n-hexane-ethyl acetate (60/40). The synthesized compounds were characterized by 1H NMR and 13C NMR spectroscopy in DMSO (Dimethyl sulfoxide)-d6 (400MHz, Bruker) using Tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer and the following scheme (Fig. 1) was employed to synthesize two novel ferulic acid derivatives

General Procedure for the Synthesis of Analogue4 (Scheme-4)

FA4 was synthesised by adding ferulic acid 0.5 gr (1eq) (SIGMA, 99% purity) and dry N-methyl amine (3eq) in the solvent (DCM and add one (or) two drops of DMF). Then addition of base and HATU (3eq 1.5 gr) to the above solution then stir the reaction for 5 hr. Check the TLC with reference of starting. Once reaction completed addition of water and use same solute for the extract. Then organic layer was washed with concentrated Hcl (0.2mol). Then after washing dried the organic layer addition of sodium sulphate and then solute was evaporated by using rota evaporator, finally the crude compound was obtained as colour less solid and Yield: 71% and the schematic reaction has represented in fig.1 (a).

Molecular Docking Studies

In present study all the calculations were carried out with high frequency computational analysis of design and optimization of lead molecules, protein ligand interaction studies by molecular docking etc., a Linux established Operating System Hi-end server (Pentium IV 3.4 MHzs, AMD Athlon 64 bit, Quadra processor with 4 GB RAM) Constructed by HCL Corporation, Pondicherry, India. The target sequences of MDM2 for anticancer activity were incurred from Protein Data Bank and the proten pdb id is 1T4F. The results of the docking study were expressed in terms of free energy of binding in kcal/mol. The ligand-protein docking complexes were analyzed by pyMOL visualizing program [6].

Preparation of Ligand

Ferulic acid and (FAA1 and FAA2) 3D structures of its analogues were generated by PRODRG [5] and pre-optimized using the MMFF94x force field. The optimized structures were used as initial conformations for the molecular docking studies.

Preparation of Proteins

The crystal structures of MDM2 [PDB: 1T4F], The protein structure for docking analysis was prepared by removing the co-crystallized ligand and water molecules (hetero atoms) from the crystal structure, adding hydrogen atoms and assigning the Kollman-All atom charges to the protein atoms. The resultant structure was saved as pdb file and used for the molecular docking experiments.

Docking with DNA

The structure of dsDNA [PDB: 1BNA] was taken from Protein Data Bank. The methodology of ligand and protein preparation is same as explained in the above section.

Docking Method

After the ligands and protein structures were prepared, molecular docking was performed. All ligand molecules were docked into the binding site of minimized structure proteins using Auto Dock4.2 software package from The Scripps Research Institute [6]. The atomic affinity potentials of each atom type in the ligand were calculated using grid based maps. The grid based energy evaluated the ligand conformation in docking simulations. The ligands were placed in auto grid covered grid map dimensions 60 Å × 60 Å × 60 Å with grid spacing of 0.375 Å. The docking protocols, Lamarckian Genetic Algorithm (LGA [7], Genetic Algorithm (GA) and simulated annealing were implemented in the Auto Dock4.2 for the calculation of ligand binding conformations. The outcomes of docking results were ranked by the binding energy (kcal/mol) and inhibition constants (Ki).

Antioxidant activities

DPPH ASSAY (1-diphenyl-2-picrylhydrazyl)

DPPH assays were performed using purified samples of test compounds as previously described by Blois [8]. Test samples were dissolved in DMSO and mixed with methanol solutions of DPPH (100 mM) in 96-well micro titer plates, followed by incubation at 370C for 30 min. The reaction mixture contains DPPH and test drug in a final concentration of 3 ml. Absorption of DPPH at its adsorption maximum 517 nm is inversely proportional to the concentration of the scavenger (test drug). The activity was expressed as inhibitory concentration 50 (IC50) The DPPH solution without sample solution was used as control. DPPH reduction was estimated at 517 nm. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group. All experiments were carried out in triplicate.

$$\% \text{ of inhibition} = [(\text{control} - \text{sample})/\text{control}] \times 100$$

Superoxide anion radical (O2 scavenging activity)

Superoxide scavenging activity of the test substance was determined by the method of modified NBT riboflavin photo reduction [9], modified by which depends on the light induced superoxide generation by riboflavin and the corresponding reduction of Nitroblue tetrazolium (NBT). The assay mixture contained different conc. of the FA analogue (FAA4) and EDTA (6mM containing 3 µg NaCN), NBT (50 µM) riboflavin (2 µM) and phosphate buffer 58 mM, pH 7.8) in a total vol. of 3 ml. The tubes received uniform illumination for 15 min and thereafter optical density was measured at 560 nm. An IC50 value was determined as the conc. that elicited the half maximal response.

% of inhibition = [(control- sample)/control] x 100

Evaluation of Antibacterial Activity

Preparation of bacterial culture:

Test tubes containing 10ml of sterile nutrient broth medium was taken. All the tubes were tightly plugged with cotton. A loop full of organism was inoculated in each tube. Then the tubes were incubated at 37°C for 6 hours.

Disk diffusion technique:

The Ferulic acid and its analogue (FAA4) was screened for their antimicrobial activity by using disk diffusion method. In this work Salmonella typhi (Gram-negative) and Staphylococcus aureus (Gram-positive - MTCC 3160) bacterial strains were used. The compounds were dissolved in DMSO at a concentration of 1mg/mL. The results of the antibacterial screening were compared with the standard antibacterial drug gentamicin. The concentration of bacterial suspensions was107 CFU/mL and Mueller Hinton Agar ((MHA) plates were used. Paper discs (5 mm) loaded with samples (10µL) were placed on the medium and the cultures were then incubated for 24h in humidified incubator at 37^0C. Inhibitory effect after 24 h incubation was recorded by measuring the diameter of zone of inhibition (cm).

Cytotoxicity Assay

The cytotoxicity was measured by MTT (3-(4,5- Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay. Briefly, K562 cells, Human chronic myelogenous leukemia cells (1x104 cells/well of 96-well plate) were treated with ferulic acid and its analogues (FA, FA1 and FA2) at the indicated concentrations for 72h, and thereafter 25µl of MTT solution (5 mg/ml in PBS) was added. After 2 h incubation, 100 µl of Extraction Buffer (20% SDS in 50% dimethylformamide) was added. After an overnight incubation at 37°C, absorbance was read at 570 nm with the Extraction Buffer as blank.

UV Visible

The UV visible spectra were obtained using SICAN 2301 Double Beam UV Spectrophotometer equipped with the 1. 0 cm quartz cells. Spectroscopic titrations were carried out at room temperature. The experiments were carried out by treating increasing concentration of drugs to the standard concentration of DNA in reference with 10 mM Tris–HCl buffer solution (pH 7. 4). The absorption maxima for each sample was read at 250–310 nm.

RESULTS AND DISCUSSION

This research work was focused on design and development of amide analogue of ferulic acid as novel anticancer agents. In-silico analysis of amide analogues of ferulic acid were done, compound obeyed Lipinski rule of five. The synthesized analogue of FA was characterized by NMR spectral analysis. The infrared spectral study for all the title compounds has been conducted to confirm the presence of functional groups assigned to them. 1HNMR and 13C NMR spectra were recorded on a Bruker 400 MHz NMR spectrometer using DMSO-d6 / CDCl3 as the solvent. The biological activity was evaluated for synthesized compound. The screening data showed significant biological activity. The new chemical entities of FA with biological properties were further developed by the modification of their functional groups. The drug ability of the synthesized derivative was predicted for their pharmacological properties using molinspiration online server and both the compounds examined in the present study showed satisfactory results. This supports their drug likeliness and ADME toxicity profile also found to be good values. The predicted physicochemical properties and ADME toxicity of compounds are depicted in Table 1 and 2 respectively and the following are the spectral data of the synthesized compound.

Table1. Physicochemical properties calculated for Ferulic acid (FA) and its analogue (FAA4) using molinspiration.

Compound	log *P*	TPSA	atoms	MW	nON	nOHNH	nrotb	nviolations	Volume
FA	3.15	46.53	19	254.28	3	1	4	0	235.42
FAA4	3.05	49.77	21	283.33	4	1	4	0	264.76

Table 2. ADMET predicted profile for ferulic acid and its analogues obtained from preADMET web server

Analog	Human intestinal absorption (%)	In vitro Caco2 cell permeability (nm/sec)	In vitro MDCK cell permeability (nm/sec)	In vitro plasma protein binding (%)	In vivo blood brain barrier penetration (C.brain/C.blood)
FA	90.603297	21.1177	228.559	50.414225	0.758419
FAA4	95.558160	38.3341	31.45	81.834582	0.24113

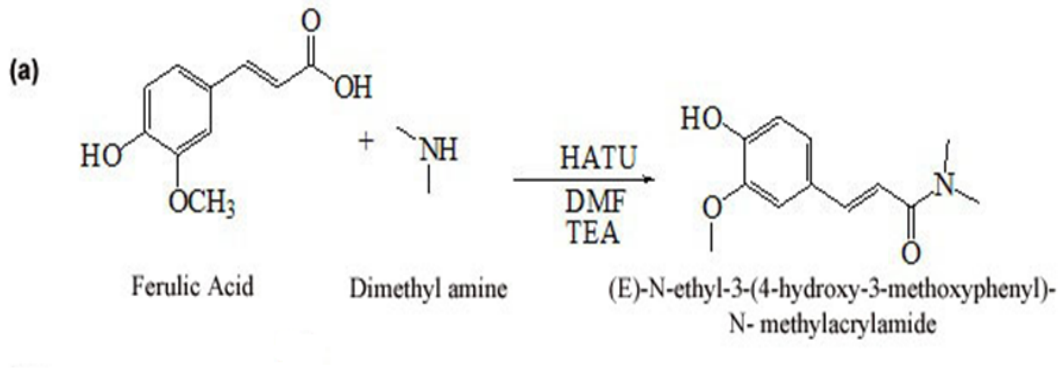

Fig. 1: Synthetic scheme of FAA4

(E)-N-ethyl-3-(4-hydroxy-3-methoxyphenyl)-N- methylacrylamide (FAA4) Spectral Data: 1H NMR (400 MHz, DMSO-d6):δ 3.300 (s,CH3, 3H); 3.713 (s, O-CH3, 3H); 6.283 (s, -C=C- Proton,1H); 6.731 (d, J = 8.4 Hz, Ar-H,1H); 6.827 (d, J = 9.2 Hz,Ar-H,1H); 6.954 (s, Ar-H, 1H); 7.312 – 7.495 (m,Ar-H,5H and -C=C-,1H); 9.573 (b, OH); 13C NMR (100 MHz, DMSO-d6): δ 38.28, 55.77, 111.65, 115.75, 121.00, 123.48, 126.00, 127.22, 128.87, 129.54, 141.27, 143.63, 147.75, 148.59, 165.25;

Docking Analysis of Ferulic Acid Derivative on MDM2 Protein

Detailed molecular docking was performed to compare the binding mode and possible interactions of ferulic acid and its analogues across MDM2 protein of Homo sapiens (homology model). The energy values were calculated by Auto Dock4.2 and characterized by intermolecular energy (consists of Vander Walls energy, hydrogen bonding energy, and electrostatic energy), internal energy of ligand and torsional free energy. The docking energy (kcal/mol), inhibition constant (μM) and the best clusters of total 100 runs for docked complexes are represented in Table 3. The docked conformations of ferulic acid and its analogue with MDM2 protein are depicted in Fig.3 and the ligand which showed hydrogen bond distance below the 5 Å were demonstrated. The obtained docking energies shows that MDM2 protein have higher affinity to ligand FA and FAA4 (Fig. 2) and as shown in Table 3, MDM2 also showed highest docking energies (-4.19 to -4.90 kcal/mol) and inhibition constants (Ki) with ligand compared to FA. The best inhibition constants shown by FAA4 (255.99 μM) were higher than compared to ferulic acid with MDM2 protein.

Table 3. Lowest binding energy (Kcal/Mol), computed inhibition constant (μM) and best cluster runs for the MDM2 with ferulic acid and its analogues determined by docking analysis.

No	Protein	Ligand	Cluster run[a]	Lowest Binding Energy[b] (Kcal/Mol)	Free Energy (ΔG)	Inhibition Constant[d] *(Ki)*[c]
1	MDM2	Ferulic acid	18	-4.19	-3.80	851.75
2		FAA4	16	-4.90	-4.44	255.99

a. The best clusters of a total of 100 runs are shown.
b .Energy value for the optimal structure in the cluster.
c. The inhibition constant (Ki) in micro molar (μM) concentration.

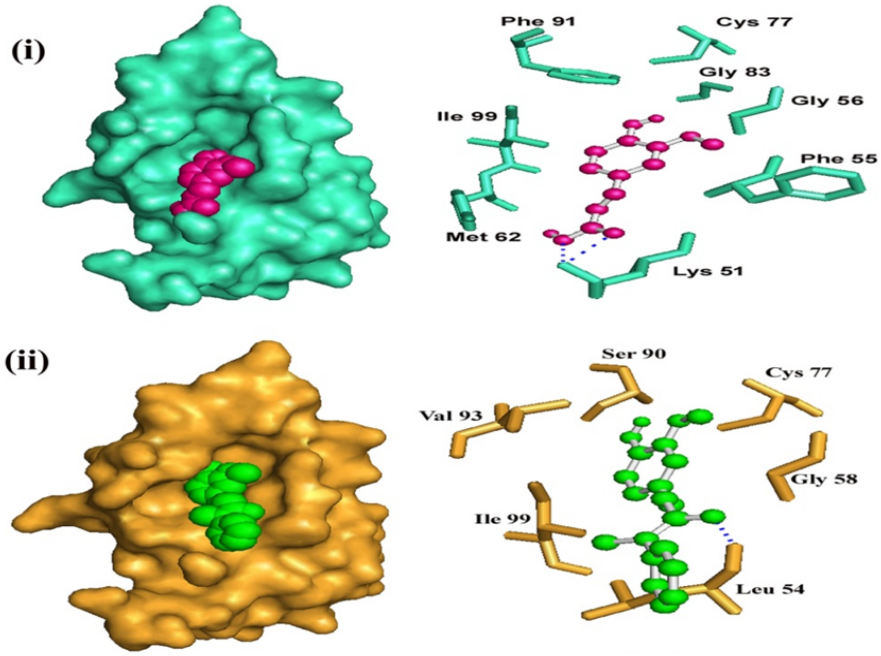

Fig. 2: (i) Binding conformation of FA hotpink spheres form onto to Mdm2 rendered green cyan, represented in surface and bound recidues in sticks and FA in ball & stics (ii) Binding conformation of FAA4 green spheres form onto to Mdm2 rendered raspberry, represented in surface and bound recidues in sticks and FA1 in ball & stics.

Antioxidant activities

DPPH free radical scavenging activity

The reduction capacity of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ferulic acid was used as standard. In these study analogues of ferulic acid exhibited significant inhibition of the lipoxygenase showing its strong potential to be developed as anti-inflammatory drug. The degree of lethality was found to be directly proportional to the concentration of the analogues. Best mortalities took place at a concentration of 200 µg/ml whereas minimal mortalities were at 10 µg/ml concentration. The IC50 values of the analogues were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the analogues the best-fit line was obtained from the data by means of regression analysis showed in Fig.4 (i). Lower IC 50 value indicates more effective antioxidant activity. The compound had good antioxidant activity and FAA4 compound was comparable with the ferulic acid showed in table.4. This significant lethality of analogues indicates the potent cytotoxic activity.

Superoxide radical scavenging Activity

The present study revealed the potent the superoxide scavenging ability of ferulic acid analogues and the IC50 values of the analogues were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the analogues the best-fit line was obtained from the data by means of regression analysis showed in Fig.4 (i). Lower IC 50 value indicates more effective antioxidant activity. The compound had good antioxidant activity showed table.4 FAA4 had more % IC50 comparing than ferulic acid showed in table.4. This significant lethality of analogues indicates the potent cytotoxic activity.

Table 4. DPPH and Superoxide radical scavenging activities of ferulic acid and its analogues

Conc. (µM)	DPPH Antioxidant assay			Superoxide radical scavenging Activity		
	AA	FA	FA4	AA	FA	FA4
10	33.74±5.3	25.37±6.7	13.65±42	15.1±3.3	13.3±4.7	6.36±.26
25	41.33±7.4	31.2±11.1	22.3±6.6	22.3±5.4	17.3±3.1	9.3±.88
50	61.4±10.3	48.6±10.3	27.6±9.1	32.5±6.3	25.3±7.7	14.6±3.1
100	75.6±15.2	62.7±15.3	38.6±10	37.8±6.7	32.2±8.1	26.6±4.5
200	83.4±14	70.6±12.4	48.7±10	45.2±7.1	37.3±8.6	29.1±8.4

DNA docking study

The docked conformation of DNA with bound molecules is shown in Fig. (2). In this docking study, the synthesized derivatives were allowed to dock with the double strand ctDNA and the structure with binding interaction was analyzed. Molecular docking analysis reveals that the compounds formed hydrogen bonds with the adenine, thymine and cytosine nitrogenous bases of the DNA. The lowest binding energies of molecules; FA and FAA4 were -4.19 and -4.27 kcal/mol respectively, FAA4 showed highest docking score with DNA. In all docking conformations, the molecules bound at major groove of the DNA (Fig. 3 (i) 1A-1B and (ii) 2A-2B. The results of docking showed that the ligands bound with DNA molecule can be stabilized by hydrogen bonding. The drugs bound with DNA in covalent/noncovalent mode possess effective anticancer activity. The results of docking interactions were found satisfactory with the spectroscopic studies

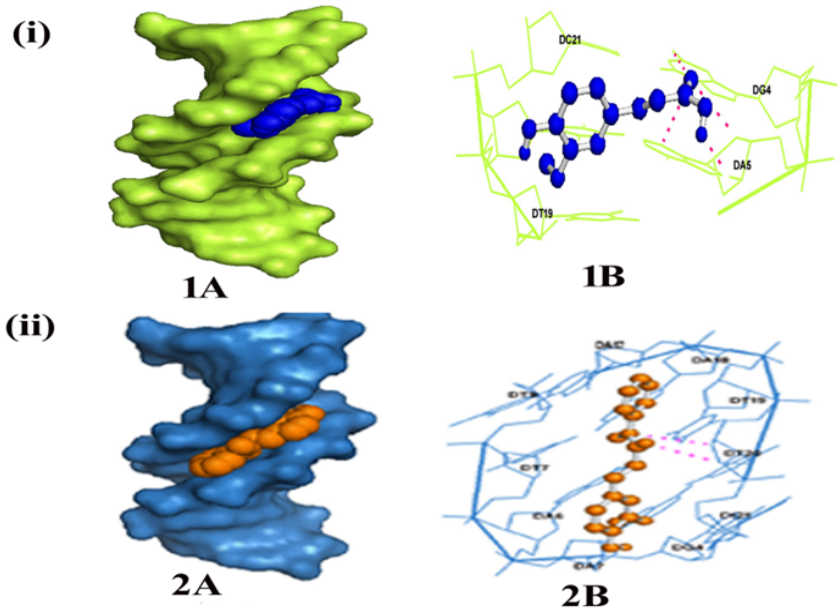

Fig.3: Schematic representation of DNA with bound compounds [(i) FA-1A & 1B and (ii) FAA4-2A & 2B]

Antibacterial activity

The results of antibacterial activity are displayed in graph Fig 4 (ii), 10µl of FA and FAA4 sample was dissolved in the 1mg/1ml of DMSO which is used for the screening. The results indicated that FA and its analogue revealed moderate antimicrobial activity against Pseudomonas aeruginosa and Staphylococcus aureus. In addition, the synthesized compound showed an effective antibacterial activity compared to a broad spectrum antibiotic, gentamicin. Moreover, FAA4 exhibited the highest antimicrobial efficacy against Pseudomonas aeruginosa and low antimicrobial efficacy Staphylococcus aureus with an inhibition zone diameter of 0.28cm and 0.39 compared to FA.

UV Visible DNA Binding Study

In DNA binding studies, the application of UV visible spectroscopy is one of the important techniques [10]. The absorption spectra of the DNA-drug interactions were depicted in Fig. 5. The absorption spectra of compounds exhibited peaks in the range 250–310 nm having absorption maxima at 260 nm. The change in absorption at 260 nm (hyperchromism) was observed upon the formation of complex compounds and the spectral changes suggest that the compounds might bind to dsDNA by interaction mode.

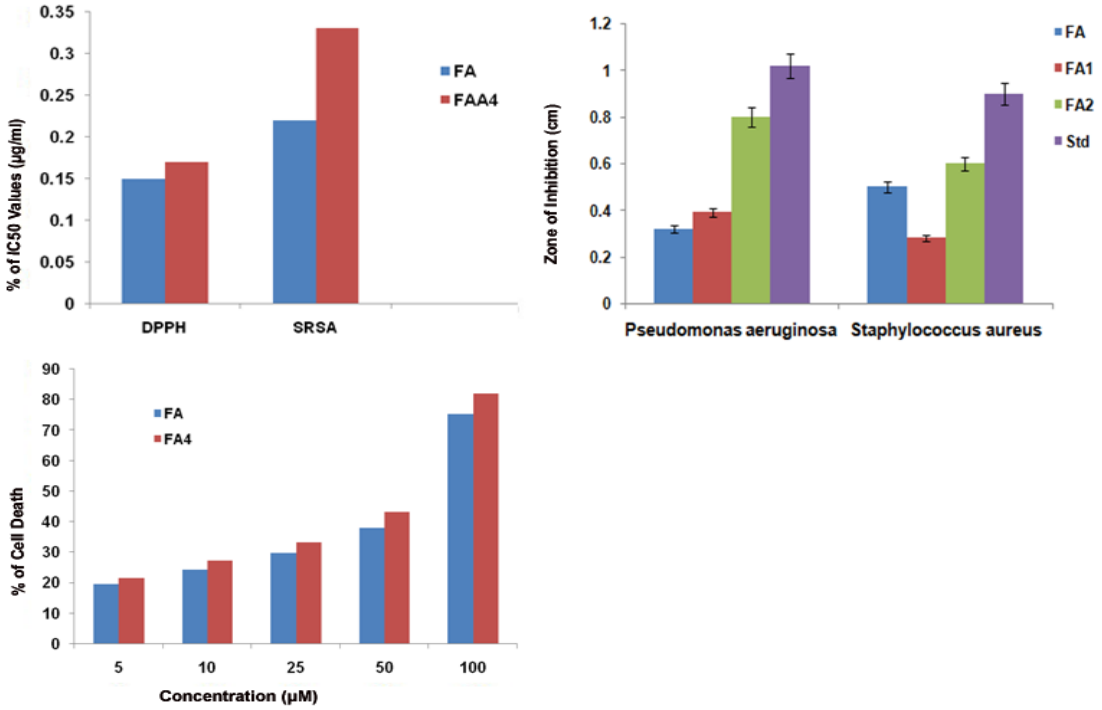

Fig.4: (i) antioxidant assay of ferulic acid and its analogues by DPPH and SRSA methods. Data represented are means ± S.D., n=3.(ii) In-vitro antibacterial activity of the ferulic acid and its newly synthesized compounds. Data represented are means ± S.D., n=3.(iii) The effect of FA and FAA4 on cell viability of K562 cancer cells. K562 cells were treated with 5, 10, 25, 50 and 100 µM concentrations of FA and FAA4 for 72 h. The percentage of viable cells with treatment was calculated in comparison with untreated control cells. The number of cells in the control was taken as 100%. Data calculated from mean absorbance ± S.D., n=3.

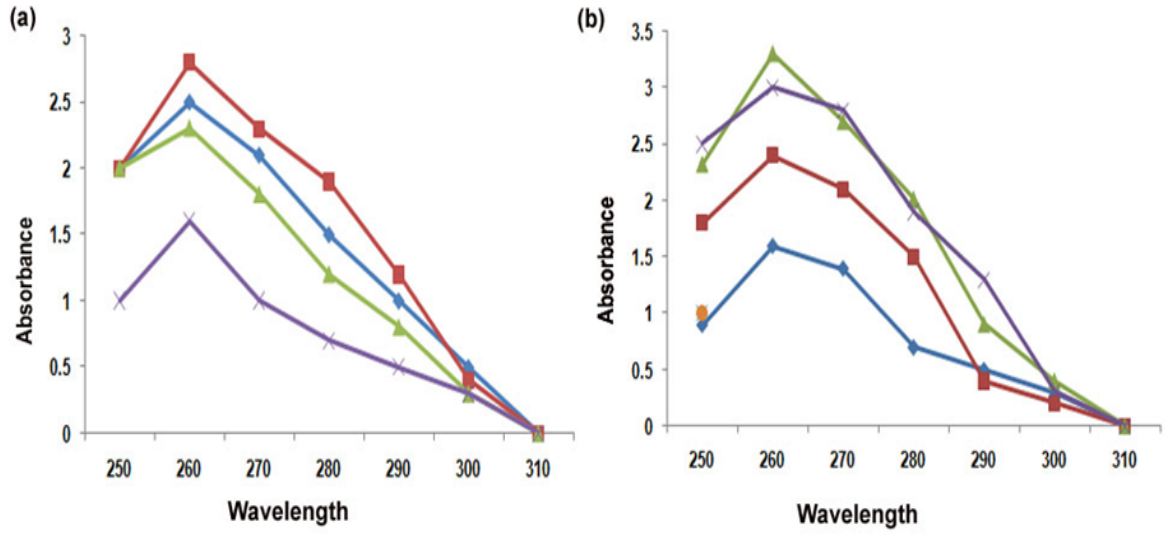

Fig.5: The UV–vis absorption spectra of DNA in free form (bottom) and bound with various concentrations of FA (a) and FAA4 (b) [compound = 0 to 25µM]. Upon increasing drug concentration, the absorbance changes were observed at 260nm is represented in arrows. Data represented are means ± S.D., n=3.

Cytotoxicity assay

Cytotoxic activity of ferulic acid and its synthesized analogues was estimated by MTT assay. Cytotoxic activity found to be increased as a positive function with the increase in the concentration of the tested of ferulic acid analogues. Among the different doses (5 µM, 10 µM, 25 µM, 50 µM and 100 µM) tested for all the compounds (FA, FA1 andFA2) at 100 µM concentration all of them have found to produce marked

inhibition on Human K562 cells proliferation. Ferulic acid has shown 80.25 % of inhibition at 100 µM concentrations Fig.4 and FAA4 showed the highest inhibition rates (82.01%). This may be due to higher rates of cell death by FAA4 compared than FA. Previous studies obtained by Naresh kumar et al [2] and Pei et al [11] also demonstrated that treatment with amide ferulic acid derivatives, yielded higher cytotoxicity

CONCLUSION

In this work, we synthesized (E) - N-ethyl-3 - (4- hydroxy- 3- methoxyphenyl) -N- methylacrylamide (FAA4) derivative as potential drug candidates against MDM2 based on the core structure of ferulic acid (FA). We carried out the receptor-ligand interactions of compounds MDM2 of human using Docking approach. The results indicated that, binding of compounds at the active site of MDM2 evidenced by the exhibition of higher docking energy and good physical interactions, thus they leads to strong evidence for the inhibition of MDM2 action and thereby increase availability of p53 for the cell. In addition, we performed the proposed DNA docking study explained that binding mode of compounds at major groove of dsDNA expressed their anti-tumor activity through modulation of transcriptional factors that help in binding to dsDNA. Lastly, compound showed moderate results with anti-oxidant, DNA binding and cytotoxicity assays. This compound also possesses anti-microbial activity against gram positive and gram negative test bacteria. Furthermore, the elucidation of the chemical synthesis of ferulic acid derivatives with low toxicity will open up path for further validation studies in in-vivo models and eventually will enable the development of viable anticancer drugs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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