Section B

Biological activities of the prepared compounds

1. Introduction

Triterpenes represent a varied and important class of natural compounds. Among these, pentacyclic lupane-type triterpenes are one of the most significant subclass which has been shown to possess several medicinal properties. Triterpenoids have been used in traditional Asian medicine for centuries.[76] Naturally occurring triterpenoid such as betulinic acid, oleanolic acid and ursolic acid are known to have antibacterial, anti-inflammatory, antitumor and antileukemic activities.[76] But the reported activities of these naturally occurring triterpenoids are comparatively weak. To increase their usefulness, researchers around the globe are involved to synthesize various different novel derivatives that have exhibited superior activities against the tested organisms or the tested diseases. In a nut shell it is clear that suitable derivatives of these naturally occurring triterpenoids have superior activities against human ailments.

To this end the present author has determined the antifungal, antibacterial and anti topoisomerase activity of the prepared compounds. Because of the very poor solubility of the pyrazine derivative of friedelin in DMSO the present author was unable to determine its biological activities.

As the pyrazine derivative of betulinic acid showed better antifungal and antibacterial activity, the present author has determined the anti topoisomerase activity of this compound.

1. Antifungal and antibacterial activity

We studied both the *in vitro* antifungal and antibacterial activity of all isolated triterpenoids. Five different fungal pathogens namely, *Colletotrichum camelliae*, *Fusarium equiseti*, *Alternaria alternata*, *Curvularia eragrostidis*, *Colletrichum gloeosporioides* were used for the antifungal study and for antibacterial study *E. Coli*, *B. Subtilis*, *S. aureus*, *Enterobactor* and some others were used as bacterial pathogen. Suitable strains of these organisms were procured from the microbiology laboratory of our institute. MICs (Minimum inhibitory concentration) of the triterpenoids against bacterial and fungal pathogen are reported in table 1& 2 respectively. DMSO (Dimethyl

sulfoxide) was used as solvent to prepare different concentrations of the triterpenoids. Solvent control (DMSO) was also maintained throughout the experiment. All experiments were performed in Petri dishes and were incubated at 37 °C for 48 h. The bacterial growth was confirmed by a change of yellow to purple colour. Bacterial nutrient media was prepared by using agar, beef extract and bacto peptone in distilled water and the pH of the solution (6.8 - 7.0) was adjusted. Culture media for fungal strains were prepared by mixing in suitable proportions of potato extract, dextrose and agar powder. All glass apparatus, culture media were autoclaved before use. The whole process was carried out in inoculation chamber. Additionally spore germination by wet chamber method was also used for determination of antifungal activity[77] (Table 3). We compared the antifungal activities of the compounds with streptomycin and antibacterial activity with ampicillin, a β-lactam antibiotic.

1.1. Results and Discussion

The antibacterial and antifungal activities of both the pyrazine derivatives were determined against four different bacterial and five different fungal pathogens. The results are tabulated in tables 1, 2 and 3. Both disc diffusion and spore germination techniques were used to evaluate the antifungal activity.

From the results as illustrated in various tables it is clear the all the compounds showed prominent antimicrobial activities against the tested fungal and bacterial pathogens as were observed from the experimental results (Table 2, 3 and 4). Compound 7, pyrazine derivative betulinic acid showed better MIC values against different bacterial strains (Table 1). Against *B. subtilis* it showed MIC values lower than 100 μg/mL. A clear comparison between compound 7 and ampicillin showed that the prepared pyrazine derivative had superior activity against *E. coli* and *Enterobactor* than the renowned ampicillin; it has lower MIC values than ampicillin (Table 1).

Table 2 MICs of compound **7** and **27** against different bacteria (Agar cup method)

Compounds	MIC in μg/mL against different strains of bacteria			
	EC	BS	SA	EB
7	100	<100	100	100
27	150	100	200	100
Ampicillin	128	64	64	128

BS- *B. subtilis*, EC- *E. coli*, SA- *S. aureus*, EB- *Enterobactor*, MIC- Minimum inhibitory concentration.

Table 3 MICs of compound **7** and **27** against different fungi (Agar cup method)

Compounds	MIC in μg/mL against different strains of fungi				
	CG	FE	CE	AA	CC
A	<5	20	40	10	<5
B	4.87	19.5	40	19.5	39
Streptomycin	1.25	2.5	<2.5	2.5	2.5

CG- *Colletrichum Gleosproides*, FE- *Fussarium equisitae*, CE- *Curvularia eragrostidies*, AA- *Alterneria alternata* , CC- *Calletotricheme camellie*.

Table 4 Evaluation of antifungal properties of reported triterpenoids against five virulent pathogens by spore germination bioassay (wet chamber method) after 48 h of incubation

Fungal pathogen	Compounds	
	7	27
	PG[a] PI AL[b] (μm)	PG[a] PI AL[b] (μm)
CC	00 100 00	05 95 4.5
FE	00 100 00	00 100 00
AA	00 100 00	00 100 00
CG	00 100 00	00 100 00
CE	05 96 06	00 100 00

CG- *Colletrichum gleosporioides*, FE- *Fussarium equisitae*, CE- *Curvularia eragrostidis*, AA- *Alternaria alternate*, CC- *Calletotricheme camellie*. PG-Percent germination, PI-Percent Inhibition, AL-Average germ tube length, [a]Based on 200 spores, [b]Based on 25 germ tubes.

The five different fungal strains used to determine the antifugal activities of the prepared pyrazine derivatives namely *Colletrichum gleosporioides*, *Fussarium equisitae*, *Curvularia eragrostidis*, *Alternaria alternate*, *Calletotricheme camellie* cause mainly wilt disease to tomato or pineapples cultivated in this part of India.

2. Antitopoisomerase activity of the synthesized pyrazine derivatives

2.1 Introduction

Each of our cells contains about 2 meters of DNA, all folded into the tiny space inside the nucleus, which is a million times smaller.[78] As one might imagine, these long, thin strands can get tangled very easily in the busy environment of the nucleus. To make things even more complicated, DNA is a double helix, which must be unwound to access the genetic information. To help with these problems, our cells build several different topoisomerase enzymes that untangle and relax DNA strands.[78]

Cellular DNA is extremely compacted, implying a high degree of structural organization. The folding mechanism not only must pack the DNA but also must permit access to the information in the DNA. The complicated structural folding pattern of DNA is commonly known as **supercoiling**. Supercoiling means the coiling of a coil. DNA is coiled in the form of a double helix, with both strands of DNA coiling around an axis. The further coiling of that axis upon itself produces DNA supercoiling. DNA super coiling is generally a manifestation of structural strain. When there is no net bending of DNA axis upon itself, the DNA is said to be in relaxed state.

DNA supercoiling is a precisely regulated process that influences many aspects of DNA metabolism. Every cell has enzymes with the sole function of underwinding and/or relaxing DNA. The enzymes that increase or decrease the extent of DNA underwinding are topoisomerase. These enzymes play an especially important role in processes such as DNA packing.

Several measurable properties of supercoiling have been established. This work has drawn heavily on concepts derived from a branch of mathematics called **topology**, the study of the properties of an object that do not change under continuous deformations. For DNA continuous deformations include conformational changes due to thermal motions or interaction with proteins and other molecules.

For any type of DNA topological change, replication or transcription (Figure 18) supercoiled DNA molecules must unwind it's supercoiling. This prerequisite unwinding of DNA supercoiling is catalyzed by the enzyme DNA topoisomerase through the catalytic TYR residue at the central DNA binding domain.

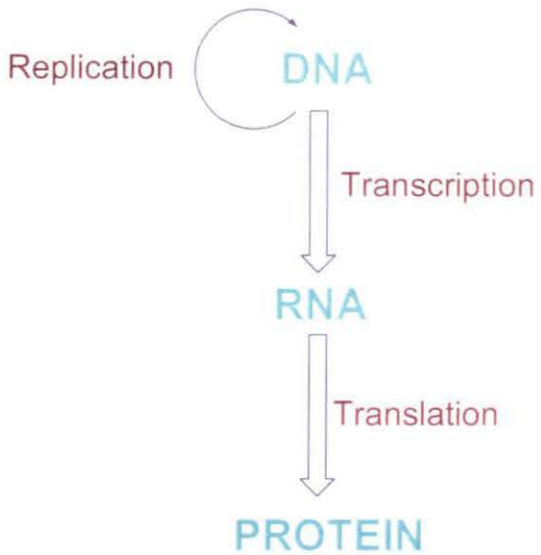


Figure 18 The central dogma of molecular biology showing the general pathway of information flow via replication, transcription and translation

Human topoisomerase I and II are ubiquitous nuclear enzymes involved in the control of DNA topology.[78-80] During the catalytic cycle, the enzyme (topo II) transiently cleaves DNA, passes an intact double helix through the break and reseals it. Vertebrates contain two isoforms of the enzyme, topo IIα and β.[78]

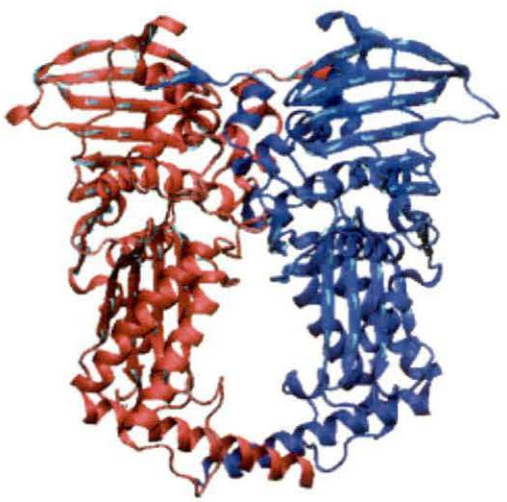


Figure 19 Complete secondary structure of topisomerase IIα

2.1.1 Relaxing DNA

Class I topoisomerases solve the problem of the tension caused during the winding and unwinding of DNA. It wraps around the DNA and makes a cut in one strand. Then, while holding onto the damaged spot, the enzyme allows the helix to spin, releasing any over winding or underwinding. Once the DNA is relaxed, the topoisomerase reconnects the broken strand, restoring the DNA double helix.

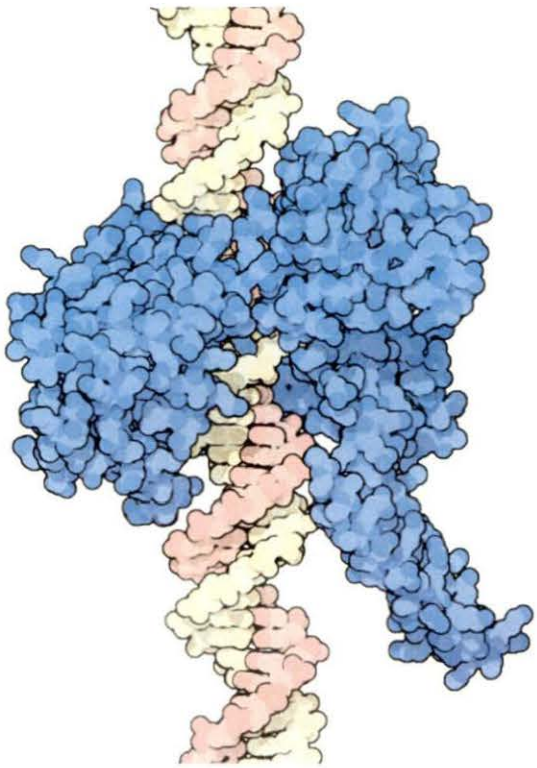


Figure 20 Schematic view of DNA bounded topoisomerase I

2.1.2 Class II Topoisomerase

Class II topoisomerase performs the amazing feat of breaking a DNA double helix, passing another helix through the gap, and resealing the double helix behind it. The picture shown here is built from two PDB entries: **1bgw**[82] has the lower part of the topoisomerase, and **1ei1**[83] is a domain from a gyrase, which is similar to the upper part of the topoisomerase. The topoisomerase is thought to be a highly dynamic structure, with several gates for entry of DNA into the two DNA-sized holes. Two tyrosine amino acids,

shown in red, cleave the DNA strands and form a covalent bond with them, holding them tightly until the DNA can be restored.

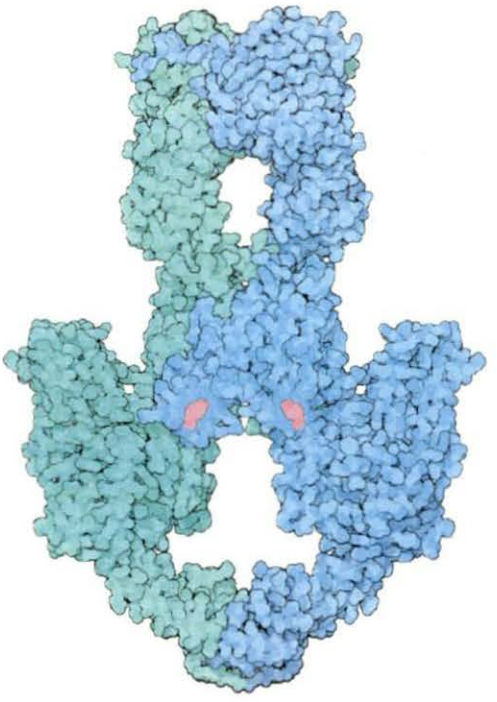


Figure 21 Complete structural model of topoisomerase II with several gates for entry of DNA

2.1.3 Untangling DNA

Class II topoisomerases are specialized in untangling DNA in the nucleus. For instance, when a cell is dividing, it needs to separate the two copies of each chromosome. During this process, portions of the two sister chromosomes may become looped around each other, getting hung up together as they are separated. Class II topoisomerase solves this problem by allowing one DNA helix to pass through the other one. It cuts both strands of one DNA double helix, keeping a firm grip on both halves. Then, it passes the other DNA through the gap, resolving the tangle. Finally, it reattaches the broken ends, restoring the DNA. The crystal structure of type II topoisomerase-DNA cleavable complex stabilized by the anticancer drug etoposide[84] is shown below. It clearly shows the number of amino acid residues for DNA binding site and cleavage core.

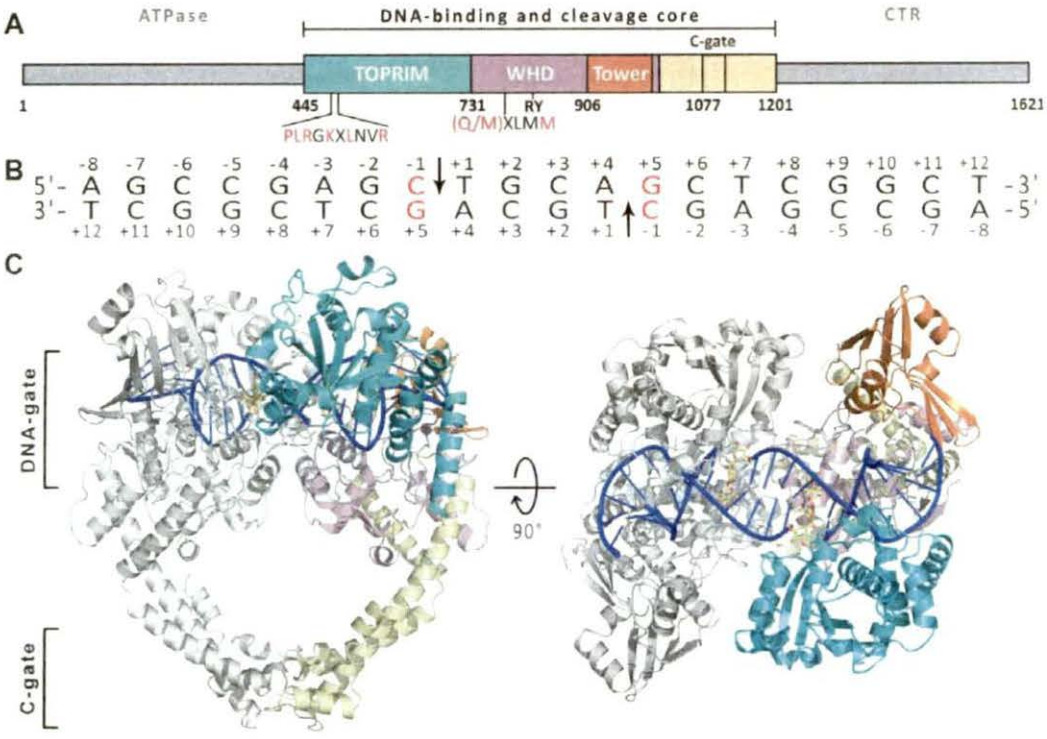


Figure 22 Structure of the hTOP2bcore-DNA cleavage complex stabilized by the anticancer drug etoposide. (**A**) Linear domain organization of hTOP2b. The middle fragment (residues 445 to 1201), corresponding tohTOP2bcore, was used in this study. The nomenclature of the TOP2 domains is adopted from the yeast enzyme.[85] Key drug-interacting residues whose mutations may confer drug resistance to antibiotics (for bacterial TOP2s) or anticancer agents (for eukaryotic TOP2s) are shown in red. (**B**) The palindromic DNA substrate used for crystallization. The cleavage sites are indicated by arrows. Positive and negative numbers (+1 to +12 and −1 to −8) designate nucleotides downstream and upstream of the scissile phosphate, respectively, with the +1 nucleotide forming a phosphotyrosyl linkage with Y821. The −1/+5 base pairs (in red) highlight the nucleotide preference for this position. (**C**) Orthogonal views of the ternary cleavage complex. DNA is in blue, one hTOP2bcore monomer is in gray, and the other follows the scheme shown in (**A**).

These processes of relaxing and untangling are essential for the proper maintenance of our DNA, so topoisomerases are sensitive targets for poisons or inhibitors. If topoisomerases are blocked, the cell will encounter problems during transcription of the DNA and during cell division. Cancer chemotherapy takes advantage of this, using drugs that block topoisomerases to kill rapidly-dividing cancer cells. For instance, the widely-used anthracycline drugs, like doxorubicin and daunorubicin, attack class II topoisomerases, and the plant toxin campothecin blocks the relaxing action of class I topoisomerases.

2.2 3D Molecular docking studies

To determine whether the pyrazine derivatives have potential as topoisomerase inhibitor, the synthesized pyrazine derivatives were initially screened for 3D molecular docking on the crystal structure of the enzyme (1bgw PDB for topoisomerase IIα)[82] using AutoDock 4 (vide infra).

From the present 3D molecular docking studies, it is clear that the pyrazine derivative of dihydrobetulinic acid binds to the central catalytic domain (please see figure 22 above) of topoisomerase IIα. The conserved amino acid residues are TYR 806, TYR 813, VAL 814, LYS 1203, TYR 709, ASN 715, PHE 712, TYR 819, ARG 793, ASP 801 and PRO 798 (Figure 23). A closer look does reveal that the molecule binds to a condensed positively charged partially hydrophobic pocket of the enzyme. It forms two direct hydrogen bond interactions, one with the oxygen atom of the neighboring ASP 801 residue through the acidic hydrogen atom of the C-28 carboxylic acid group another with the oxygen atom of TYR 813 through the aromatic π-cloud of the pyrazine moiety.

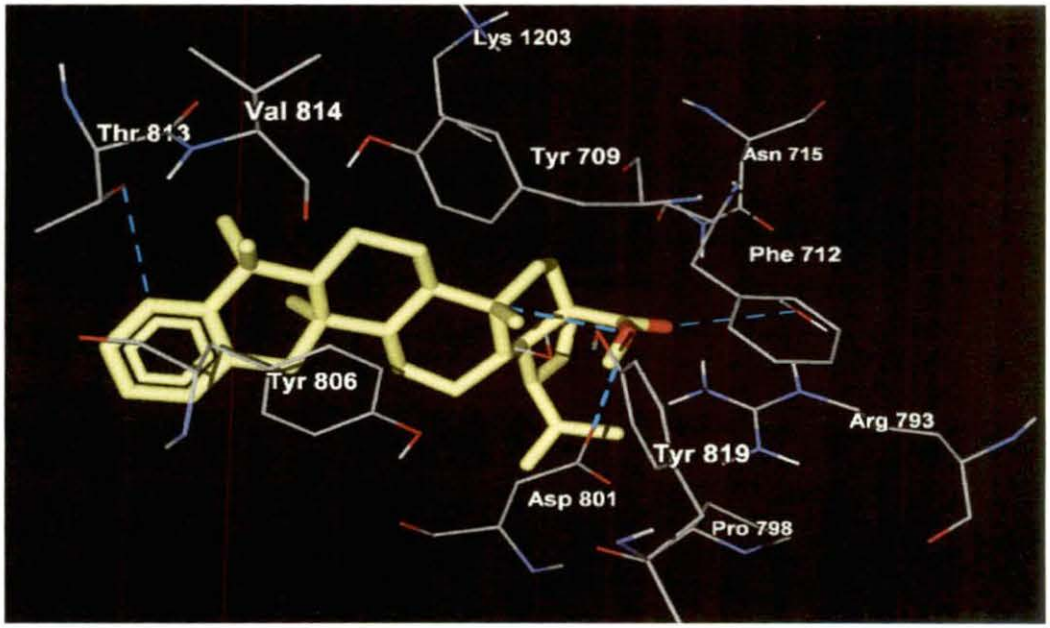


Figure 23 H-bond interaction view of the pyrazine derivative of dihydrobetulinic acid

In addition to this, it forms another one water mediated H-bond interaction to the π-cloud of PHE 712 involving the carbonyl oxygen of the C-28 carboxylic acid group.

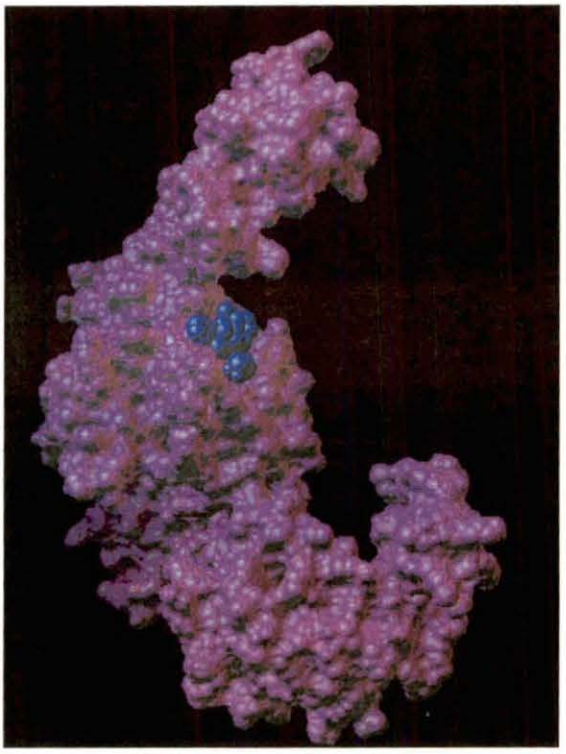
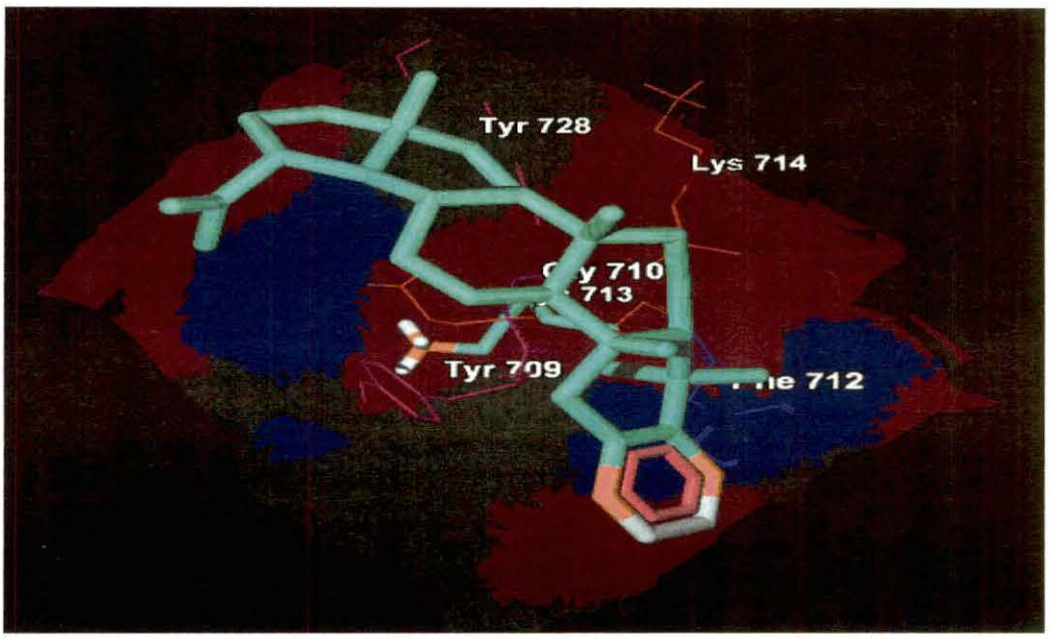


Figure 24 Detailed docked view of pyrazine derivative of dihydrobetulinic acid showing that the molecule has binded to one of the two possible gates for DNA binding

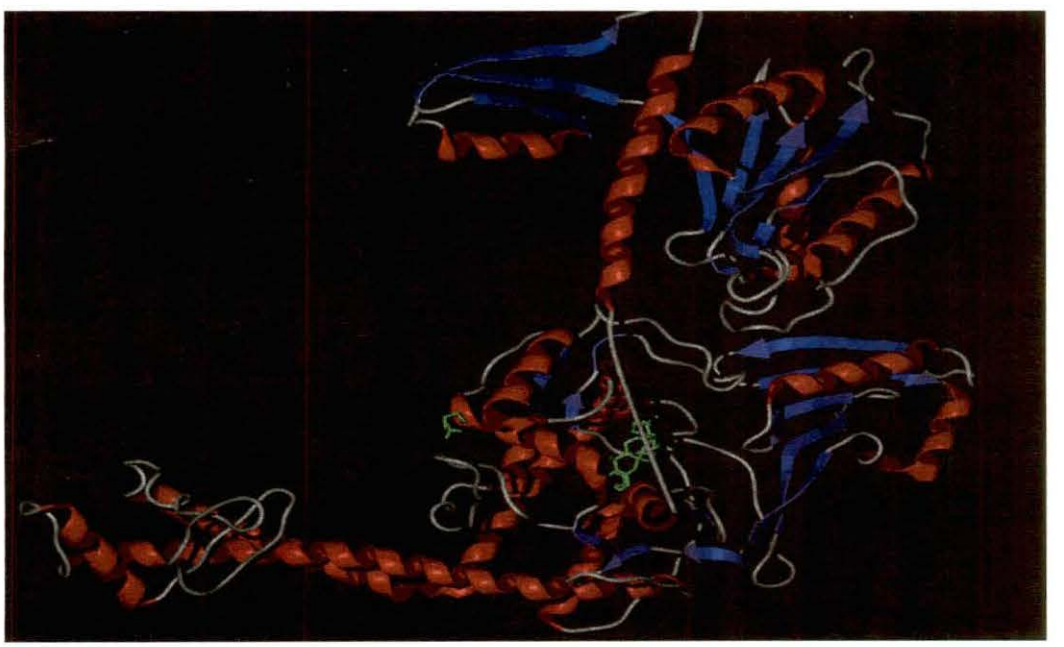
It was also interesting to note from the present docking studies that the synthesized pyrazine derivative has binded to the upper gate for DNA binding thus clearly inhibiting the further binding of DNA to the enzyme.

On the other hand the pyrazine derivative of lupeol binds to a condensed positively charged hydrophobic interior (Figure 25A and 25B) of the enzyme. It does not show any covalent bonding to a definite site of the binding pocket of the enzyme. It does not form any H-bond interaction with the any amino acid residues. The conserved amino acid residues that are in the vicinity are TYR 809, TYR 728, LYS 713, GLY 710, LYS 714 and PHE 712. Figure 25B shows the secondary interaction docked view; it is clear from

the view that there is no hydrogen bond between the synthesized pyrazine derivative and the neighboring amino acid residues.



(A)



(B)

Figure 25 Detailed docked view of the pyrazine derivative of lupeol, (**A**) Hydrophobic interaction view and (**B**) Secondary structure view.

Therefore the type of interaction between the ligand molecule and the host molecule must be either electrostatic interaction or dipole induced dipole interaction. It showed the binding energy of -9.76 kcal/mol.

Similar to these facts the prepared pyrazine derivative of friedelin does not show any H-bond interaction (Figure 26) with the enzyme 1bgw, pdb. It also binds to a hydrophobic pocket of the enzyme comprising of CYS 712, LYS 714, LYS 713, GLY 710, TYR 728 TYR 709 residues and the estimated binding energy value is -9.22 kcal/mol. Therefore the nature of interaction here also must be either electrostatic or dipole induced dipole interaction.

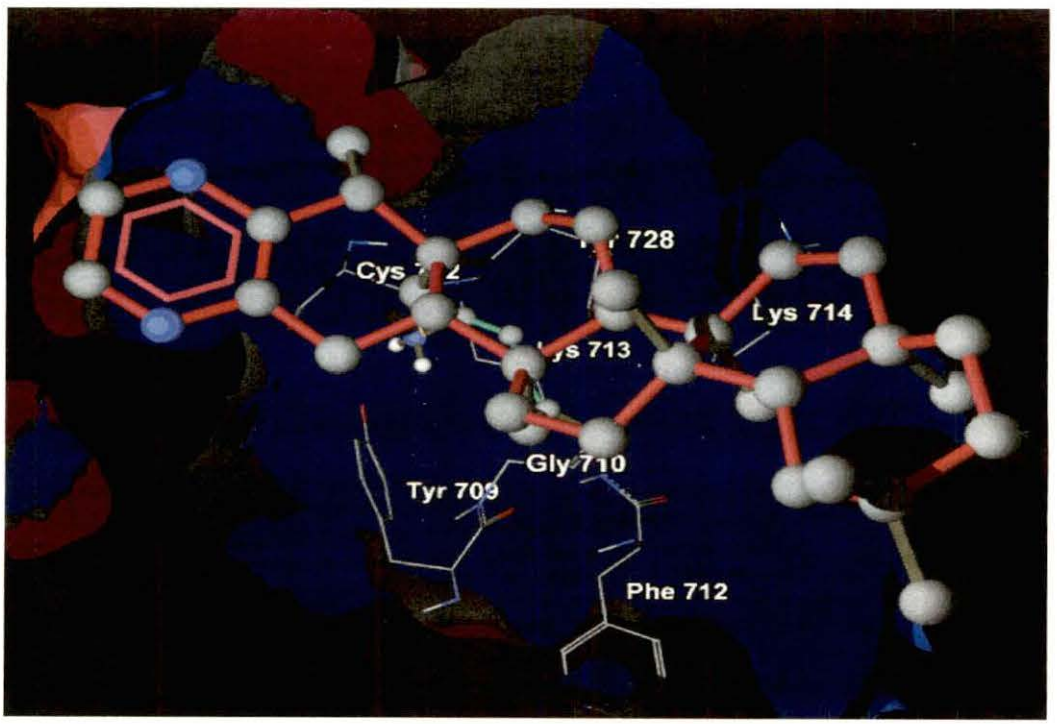


Figure 26 Detailed docked electrostatic interaction view of the pyrazine derivative of friedelin.

From the figure it can be said that the synthesized lead molecule can bind to the catalytic domain i.e. the DNA binding domain of the enzyme molecule. Except lysine all other neighboring amino acid residues are neutral in character. Another interesting fact is that the phenolic –OH group of TYR 709 turned inside towards the aromatic pyrazine moiety at the ring A of the pentacyclic skeleton. It might be possible that the expected nonionic

interaction is operating through that phenolic –OH group and with the aromatic ring current (Figure 26).

From the present docking studies on the crystal structure of topoisomerase IIα (1bgw, pdb) it was clear that all the synthesized pyrazine derivatives can bind to the DNA binding domain of the enzyme. Thus it can be infer that all the three derivatives can inhibit topoisomerase IIα to bind with DNA.

Of the three pyrazine derivatives, that of betulinic acid showed the best form of results as it binded to the upper DNA binding gate and it is the only compound that showed the important H-bond interaction with the enzyme. These attractive findings encouraged the present author to determine the experimental topoisomerase IIα inhibitory activity of this compound.

2.3 Topoisomerase inhibitory activity of pyrazine derivative of dihydrobetulinic acid

Inhibition of catalytic activity of topoisomerase constitutes a useful strategy for the identification of potential antitumor agents. Topo IIα creates transient breaks in supercoiled DNA resulting in DNA relaxation. The relaxed DNA can be distinguished from supercoiled DNA by gel electrophoresis analysis. The results of molecular docking studies on interaction of pyrazine derivative of dihydrobetulinic acid with DNA binding domain of human Topo IIα, was further confirmed by examining DNA relaxation activity of Topo IIα. The ATP dependent relaxation of supercoiled DNA by the enzyme was monitored in absence or presence of pyrazine derivative of dihydrobetulinic acid. From the results in figure 27, it is evident that the inhibitory effect of the compound was dose dependent. They showed complete inhibition of the catalytic activity of Topo IIα at 100 and 50 μM concentrations, whereas partial inhibition of the activity was observed at a concentration of 25μM.

The pyrazine derivative of methyl dihydrobetulanate was hydrolyzed successfully to the corresponding acid and to that the experimental work of DNA topoisomerase inhibition activity was carried out.

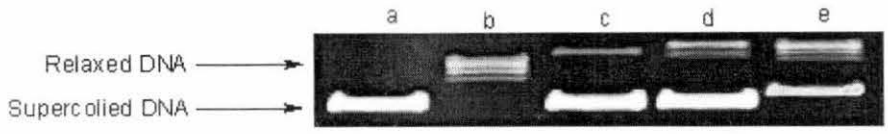


Figure 27 Effect of pyrazine derivative of dihudrobetulinic acid on DNA relaxation activity by DNA topoisomerase IIα, Lane a: superolied DNA alone, Lane b: control no drug (with 2 units of topo II), Lane c, d and e: 100, 50 and 25 mM concentration of the synthesized pyrazine derivative respectively.

3. Conclusion

The present author has successfully synthesized the pyrazine derivatives of lupanol, methyl dihydrobetulanate and friedelin. All the molecules were characterized by spectral data and by comparison to that reported in literature. 3D molecular docking of these derivatives in the central catalytic domain of topoisomerase IIα (1bgw PDB for topoisomerase IIα) revealed the nature of the binding and the type of interactions between the synthesized compounds and the enzyme. The topoisomerase IIα inhibitory activity was further confirmed by *in vitro* experiments. The present work will definitely enrich the modern drug designing towards the invention of newer plant based chemotherapeutics to fight against human ailments. In addition the findings may provide a better understanding of the structure activity relationship towards the topoisomerase inhibitory activity of the pentacyclic triterpenoids.