

## Investigated the Interaction between Terpenoids of Betulinic Acid and Ursolic Acid Binding with (Ct-(Ds) DNA) Was Studied By Spectroscopic, Electrochemical and Molecular Modeling Approach

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**Abstract:** The interaction between terpenoids of Betulinic Acid (BA) and Ursolic Acid (UA) binding with calf thymus-double strand deoxyribose nucleic acid (Ct-(ds) DNA) was studied by employing UV absorption, fluorescence, cyclic voltammetric and molecular modeling techniques. All studies were confirmed that the structural changes of DNA were binding to the terpenoids. From the CV results positive shift in peak potential and increased peak current of the terpenoid in the presence of DNA and then the fluorescence quenching of DNA-terpenoids system indicated the intercalative mode of binding between flavonoid and DNA. Molecular docking simulation methods are used as tools to delineate the binding mode and probable location of the terpenoids and their effects on the stability and conformation of Ct-(ds) DNA. Furthermore, Betulinic acid can bind more potential with Ct-(ds) DNA than ursolic acid. This is helpful to understand the molecular aspects of binding mode and provides direction for the use and the design of new effective therapeutic agents, especially for anti-bacterial, anti-malarial, anti-inflammatory, antioxidant and anti-cancer activity.

**Keywords:** Terpenoids; Ct-(ds) DNA; Interaction; Spectroscopy; Cyclic voltammetry; Docking.

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### Abbreviations:

BA	-	Betulinic Acid
UA	-	Ursolic Acid
DNA	-	Deoxyribose Nucleic Acid
Ct	-	Calf Thymus DNA
(ds)	-	Double Strand DNA
Tris-HCl	-	Tris (hydroxymethyl) aminomethane
DMSO	-	Dimethyl Sulphoxide

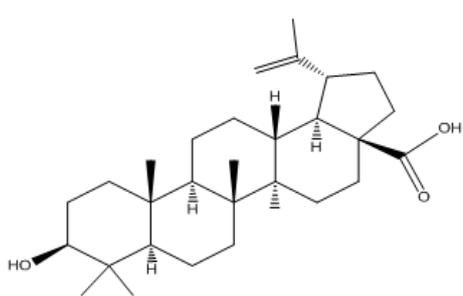
### I. Introduction

The mechanisms by which natural compounds protect against different stressors are complex and varied. Individual phytochemical has been implicated as modulators of many metabolic and regulatory processes. Triterpenoids are a class of naturally occurring compounds that are found in a variety of plants [1]. Terpenoids are the secondary metabolites obtained naturally and major constituents present in fruits, vegetables, spices and are formed by 2-methylbutane residues, less precisely but usually also referred to as isoprene units (C<sub>5</sub>H<sub>8</sub>) and called as isoprenoids known to build up the carbon skeleton of terpenes.

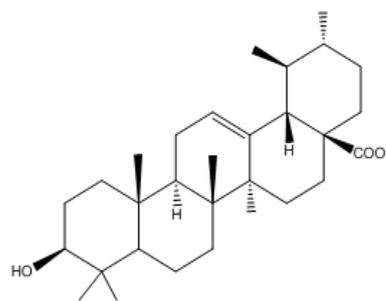
Osmium is one of the most important genres of the Lamiaceae family, due to the extensive use of many of its species as economically important medicinal and culinary plants. Some people from Brazil have been using infusions of Ocimum species for ritualistic aromatic baths, and as a tea for treating gastro-intestinal problems and also for seasoning special foods. Terpenoids are broadly classified on the basis of the number of isoprene units present in the molecule.

Ursolic acid was previously identified in only two species of Ocimum: O. basilicum and O. tenuiflorum. Now the ursolic acid content in eight species of the genus Ocimum: O. americanum, O. basilicum, O. basilicumvar purpurascens, O.basilicumvar minimum, O. gratissimum, O. micranthum, O. selloi and O. tenuiflorum grown in the Northeast of Brazil [2,3]. Although originally thought to exhibit specific cytotoxicity

against melanoma cells, this agent has been found to be cytotoxic against non-melanoma tumor cell types including neuroectodermal and brain tumor cells.



**Figure: 1.A (Betulinic acid)**

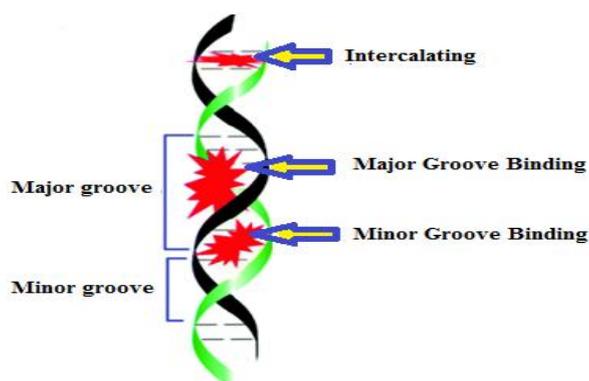


**Figure: 1.B (Ursolic acid)**

BA and UA are the two major pentacyclic triterpenoids studied in many other plants, individually or in groups including genus *Ocimum*. These pharmacologically active triterpenoids have gained worldwide attention due to their promising therapeutic index. Reports suggest antioxidant, anti-carcinogenic, anti-ulcer, hepatoprotective, anti-HIV, and anti-malarial properties of these triterpenoids (BA and UA) in various plants including *Ocimum* species<sup>[4]</sup>. Ursolic acid (UA) (3 $\beta$  hydroxy urs-12 en-28 oic acid) is one such a triterpenoid found in plants like *Ocimum americanum*. It has been reported to possess a wide range of pharmacological properties<sup>[5]</sup>. Betulinic acid, (3 $\beta$ -hydroxy-lup-20(29)-en-28-oic acid) is a naturally occurring pentacyclic lupane-type triterpenoid which exhibits a variety of biological and medicinal properties such as inhibition of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antinociceptive, anti-HSV-1, and anticancer activities. Betulinic acid is widely distributed throughout the plant kingdom<sup>[6]</sup>.

Over the last few decades structure of DNA and its interaction with different bioactive molecular moieties have gained a great interest in the field of organic synthesis and pharmacology. DNA is a nucleic acid that contains all the information necessary for specifying the biological development of all living bodies. It is a molecule that controls hereditary information transferred to the offspring. During reproduction, DNA is replicated and transmitted to the new trait. In this process, the sequence of DNA base pairs defines the characters of individuals ranging from physical traits to disease susceptibility. It is necessary to understand at molecular level gene expression and their mechanism of transfer to offspring<sup>[7-9]</sup>. This could be helpful to understand the transfer of many diseases. It is also a key step towards the development of new chemotherapeutic strategies. The interaction of many naturally occurring compounds with DNA adducts is an active area of research in chemistry and biology which leads to the understanding of drug-DNA interaction and the consequent design of new efficient drugs targeted to DNA<sup>[10]</sup>. Due to the central role of DNA in replication and transcription, DNA has been a major role for antibiotic, anticancer, antiviral and anti-inflammatory drugs.

Interaction of small molecules and DNA are mainly of two types. One is covalent interactions and another one is non-covalent interactions. Three major modes of non-covalent interactions are electrostatic interactions, groove binding (minor & major) and intercalative binding. The small molecule can interact with DNA involving a single mode of binding or mixed binding modes. DNA is antiparallel double helix held together by hydrogen bonding interactions between DNA base pairs. It is worth noting that the property of mixed binding mode can be linked to their mechanism of action and therapeutic efficiency<sup>[11,12]</sup>.



**Figure: 2 DNA Binding.**

The present paper attempts to understand the mechanism of binding of two terpenoids with DNA by employing spectroscopic, electrochemical and molecular modeling techniques. From these result, UV-vis spectroscopy, fluorescent spectrometry, voltammetry and molecular modeling (using Autodock 4.2) are employed, and the results could provide useful pharmacological and toxicity information.

## **II. Experimental materials and methods**

### **2.1. Materials:**

Betulinic acid and Ursolic acid were extracted from *Ocimum americanum*. These terpenoids were prepared in 10% DMSO. Calf thymus-(ds) DNA was purchased from Sisco Research Laboratories Private Limited (SRL), India and used without any purification. Stock solutions of DNA and terpenoids were prepared by dissolving in Tris-HCl buffer (pH 7.4) and double distilled water, separately. Both DNA and Terpenoids solutions were stored at 4 °C until the end of the experiment. The concentration of DNA was determined by spectrophotometrically using the extinction coefficient value of 6600 L mol<sup>-1</sup> cm<sup>-1</sup> at 260 nm. The solution of DNA was found to be free from protein as evident from its absorbance ratio value in the range of 1.7.

### **2.1.2. Methods**

#### **UV absorption studies:**

UV-vis spectra were recorded by using Agilent diode array spectrometer (Agilent 8453) at room temperature (25 °C). UV absorption spectra of terpenoids in the absence and presence of increasing concentrations of DNA were recorded in the wavelength range of 250-500nm. Matched quartz cells of 1 cm path length were used in this study. Tris-HCl buffer (pH 7.4) was used as the reference. During optical titration of the terpenoids, an equal amount of DNA was added to both the sample and the reference cells. The temperature was maintained at 4°C especially for Ct-(ds) DNA. In the spectrophotometric titrations to a fixed concentration of terpenoid, the concentration of DNA was varied and the change in the absorption at  $\lambda$  max of the terpenoid was noted at each P/D [DNA/terpenoid molar ratio]. The purity of DNA was verified by monitoring the ratio of absorbance at 260/280 nm (A<sub>260</sub>/A<sub>280</sub>). The concentration of DNA stock solution was determined according to the absorbance at 260 nm by using an extinction coefficient of 6600 mol<sup>-1</sup>.cm<sup>-1</sup>. Appropriate blanks were run under the same conditions and subtracted from the sample spectra.

#### **Fluorescence Spectral analysis:**

Fluorescence measurements were carried out on a Cary Eclipse fluorescence spectrophotometer. Measurements were made in a fluorescence free quartz cell of 1 cm path length. The fluorescence characteristics of terpenoids ( $\lambda_{ex}$  = 380 nm and  $\lambda_{em}$  = 450 nm) were used to investigate Ct-(ds) DNA-terpenoids interaction in Tris-HCl buffer solution (pH = 7.4) at room temperature. Fluorescence spectra were recorded in the range of 300-500 nm while maintaining the constant concentration of terpenoids and varying concentrations of DNA. Terpenoids were binding to DNA leading to a marked increased or decreased in fluorescence emission intensity also agrees with observations for other intercalators.

#### **Voltammetric studies:**

The electrochemical behaviors of terpenoids were studied before and after adding DNA through cyclic voltammetry (CV) using Tris-HCl buffer solution of pH 7.4 as supporting electrolyte. The mixed terpenoids-DNA solution was allowed to equilibrate for 2 min at room temperature. Furthermore, the voltammetric behaviors of both terpenoids and terpenoids-DNA adduct were studied by addition of increasing amount Ct-(ds) DNA. During the determination, a nitrogen atmosphere was maintained over the solutions.

#### **Molecular Docking Study:**

Auto Dock (4.2) with MGL Tools (1.5.4) was used for docking evaluation between Ct-DNA and Triterpenoids. The duplex dodecamer nucleotide sequence Ct-DNA (CGCGAATTTCGCG)<sub>2</sub> retrieved from the Protein Data Bank (PDB ID: 1BNA).

The ligands Betulinic acid and Ursolic acid were modeled using ChemDraw and transformed into PDB format using online OPENBABEL. The LGA (Lamarckian genetic algorithms) and all other default parameters as fulfilled. The ligands and DNA were enclosed with grid points 112×60×112 and spacing of 0.513 Å. In Autodock 4.2, ten docking runs were carried out separately and visualized using Pymol graphics programme (academic free version).

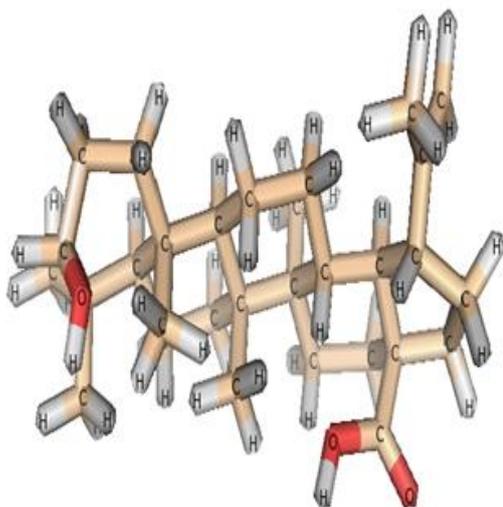


Figure: 3.A Betulinic acid

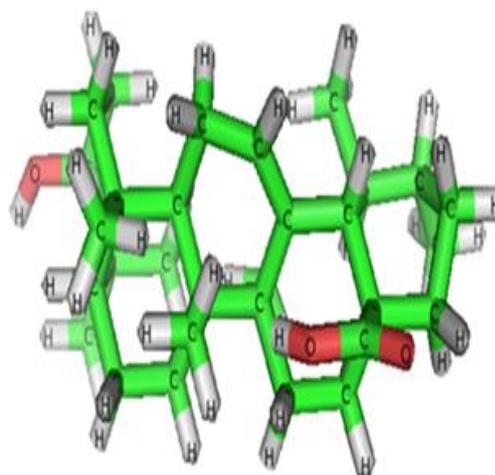


Figure: 3.B Ursolic acid

### III. Result and Discussions

#### Absorption spectroscopy:

The application of absorption spectroscopy is one of the most useful techniques in DNA-binding studies. Here, absorption spectra were obtained by (1mM) terpenoid was binding with double strand DNA (ds-DNA) solution. The results are shown in Figure 4.A, 4.B shows a single absorption band of 265 nm for terpenoids in the absence of Ct-(ds) DNA. DNA concentration was increased in the terpenoids with increased the intensity of the absorption band. Because of compound binding to DNA, the absorbance spectrum showed hyperchromism, which involve a strong stacking interaction between an aromatic chromophore and the base pairs of DNA <sup>[13]</sup>. As shown in Figure 4. A&B, the absorption peak of DNA at 265 nm exhibited gradual increased broad peak and slight red shift (Bathochromic) with the increasing concentration of Ct-(ds) DNA. Terpenoids were binding to DNA through intercalation was characterized by a change in the absorbance (Hyperchromic shift) and redshift in wavelength, due to the intercalative binding mode involving a stacking interaction between the DNA base pairs <sup>[14]</sup>. Hyperchromic shift in the spectra of terpenoids was observed evidencing helical ordering of terpenoids in the DNA helix. These spectra were compared with those calculated from the sum of absorbance of free terpenoids and the free DNA at their different concentrations. If Beer's law was strictly followed, this simulated set of spectra and the measured ones should coincide.

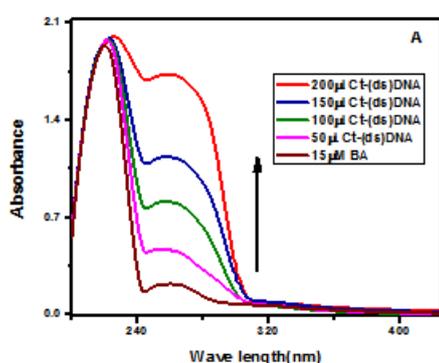


Figure: 4.A

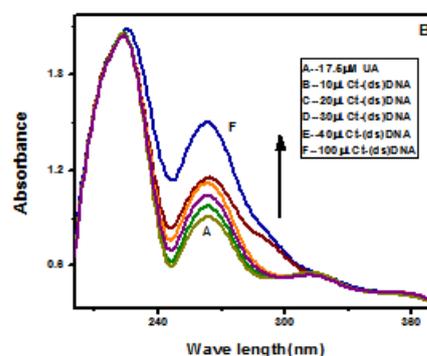


Figure: 4.B

Figure 4. Absorption spectra of (A) 15  $\mu\text{M}$  of BA and (B) of 17.6  $\mu\text{M}$  of UA with varying concentration of Ct-(ds) DNA.

This observation indicated that a stable DNA-terpenoids complex was formed. The absorption intensity at 265 nm increased significantly, and the absorption peak shifted to 280 nm, which were likely attributed to the spectral overlapping between terpenoids and DNA. The hyperchromic effect in UV spectra upon small molecule binding to DNA is a typical characteristic of an intercalating mode. The results implied that the binding mode between terpenoids and DNA might be intercalation.

On the basis of variations in the absorption spectra of DNA upon binding to terpenoids, this equation can be utilized to calculate the binding constant<sup>[15, 16]</sup>,

$$\frac{A_0}{A-A_0} = \frac{\epsilon G}{\epsilon_{H-G} - \epsilon G} + \frac{\epsilon G}{\epsilon_{H-G} - \epsilon GK} \frac{1}{[DNA]} \rightarrow 1$$

Where “A<sub>0</sub>” and “A” are the absorbances of the complex in the absence and presence of DNA, respectively, and ε<sub>G</sub> and ε<sub>H-G</sub> are their absorption coefficients, respectively. The binding constants were calculated from the ratio of the intercept to the slope of the linear fitting of the curve obtained by plotting 1/(A-A<sub>0</sub>) versus 1/[DNA] for each terpenoid. The plot of A<sub>0</sub>/(A - A<sub>0</sub>) versus 1/[terpenoid] was constructed by using the absorption titration data and linear fitting, yielding the binding constant, K<sub>b</sub>= 2.18 ×10<sup>-5</sup>mol<sup>-1</sup> L (Betulinic acid) and K<sub>b</sub>= 3.16 ×10<sup>-5</sup>mol<sup>-1</sup> L (Ursolic acid).The absorption spectra of BA and UA, when titrated with Ct-(ds)DNA, showed the isosbestic point at 300 nm and 320 nm respectively<sup>[17]</sup>.

The binding constants were calculated from the ratio of the intercept to the slope of the linear fitting of the curve obtained by plotting 1/(A-A<sub>0</sub>) versus 1/[DNA] for each terpenoid.

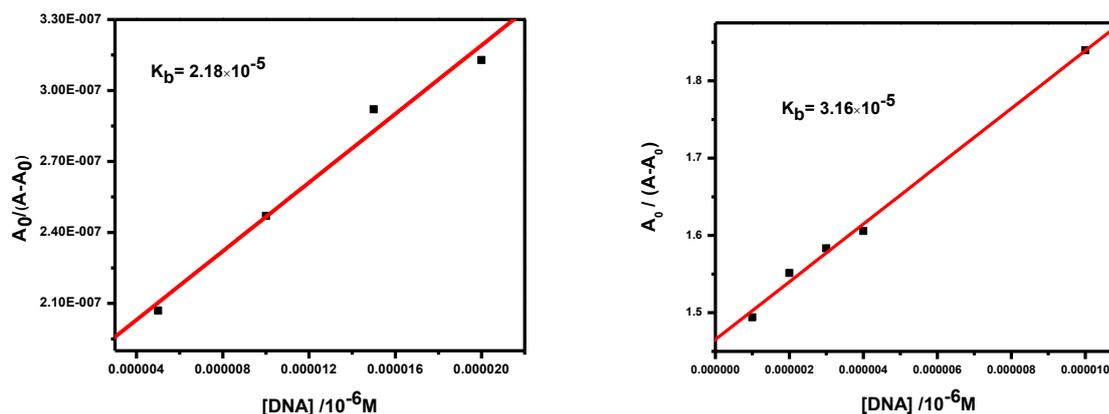


Figure: 4.A and 4.B. Fitting of experimental data of BA and UA with Eqn. (1).

The result of fitting the experimental data with equation (1) is suggested that the complex of terpenoids with DNA is to be a kind of 1:1 ratio. From a plot of A<sub>0</sub>/(A-A<sub>0</sub>) vs. 1/[DNA], the ratio of the intercept to the slope gives the binding constant, K<sub>b</sub>= 2.18 ×10<sup>-5</sup>mol<sup>-1</sup> L(Fig:4.A) and K<sub>b</sub>= 3.16 ×10<sup>-5</sup>mol<sup>-1</sup> L(Fig:4.B) .

### Emission spectroscopy:

In general, fluorescence spectroscopy has been applied in many chemical-related fields. However, the presence of Rayleigh and Raman light scattering phenomena interfere with and complicate the analysis of the classical fluorescence measurements. The synchronous fluorescence spectroscopy is an approach that can eliminate or reduce this problem significantly<sup>[18]</sup>. As a preliminary investigation, we have studied the fluorescence quenching of the synthesized compounds (Betulinic acid and ursolic acid) binding with Ct-(dsDNA. Intercalation between DNA bases and is generally used as a fluorescent probe in DNA-binding studies. Since BA & UA are aromatic molecules, the intercalation mechanism may be due to π-π and hydrophobic interactions between the aromatic ring of the compound and the DNA base. The fluorescence intensity decreased steadily with the addition of increasing concentrations of the Ct-(ds) DNA (10 to 50μM), indicating the fluorescence quenching activity<sup>[19]</sup>.

The stronger enhancement in fluorescence intensity of Betulinic acid with DNA may be largely due to the increase of the molecular planarity of the complex and the decrease of the collisional frequency of the solvent molecules with the complex which is caused by the planar aromatic group of the complex stacks between adjacent base pairs of the DNA. Terpenoids were binding to DNA leading to a marked increase in fluorescence emission intensity also agrees with observations for other intercalators<sup>[20]</sup>. In the presence of DNA, emission quenching of flavonoids may be caused by the fact that, flavonoids being a small hydrophobic molecule and can be absorbed by hydrophobic groups on the surface of DNA<sup>[21]</sup>. The fluorescence signal of 382 nm was responsible for Betulinic Acid and 371nm for Ursolic Acid. When the DNA solution was added to

the terpenoids, Betulinic Acid peak shifted from 382nm to 399nm and ursolic Acid was shifted from 368nm to 386nm. This information indicates that terpenoids were binding (quench) with Ct-(ds) DNA. Figures 5.A @ 5.B shows the fluorescence spectra of terpenoids in the presence and absence of calf thymus DNA.

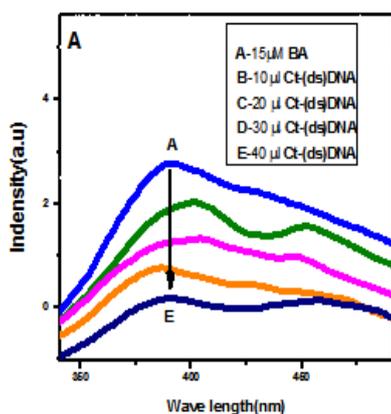


Figure: 5.A

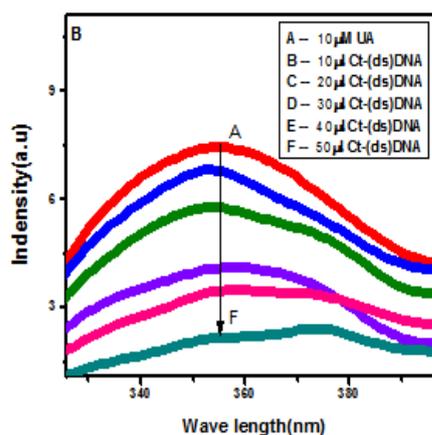


Figure: 5.B

Figure 5. Emission spectrum of terpenoids in presence of increasing amount of Ct(ds)DNA, (A) BA and

Figures: 5.A @ 5.B shows the fluorescence emission spectra of Terpenoids with various concentrations of the DNA in pH 7.4 Tris-HCl buffer solution. The emission intensity was decreased regularly with the addition of DNA concentration. In emission spectra, both compounds have to bind (turn off) character. By comparing Fig: 5.A with Fig: 5.B, we can deduce that Betulinic acid has stronger emission intensity than Ursolic acid.

#### Cyclic voltammetry:

In a cyclic voltammetry experiment, scanning the potential in both directions provides with the opportunity to explore the electrochemical behavior of species generated at the electrode. Electrochemical behavior of GCE was carefully investigated in Tris-HCl buffer by cyclic voltammetry. The electrochemical response of terpenoids in DNA solution is a rich source of information about binding and reactivity [22]. The cyclic voltammograms of terpenoids in the presence of different amounts of DNA were recorded in Tris-HCl buffer solution pH 7.4 and are shown in Fig: 5.A and 5.B. The voltammograms of BA and UA showed a prominent oxidation peak at 0.25V and 0.8V, respectively. These peaks were found to be shifted towards positive potential (from 0.25 V to 0.36 V for BA and from 0.8 V to 0.98 V for UA) in the presence of DNA.

If the electron transfer was not blocked, then the current should increase but peak shift would be expected. The observed shift in the peak potential and increased peak current was attributed to the formation of the DNA-terpenoid complex through the intercalative mode of binding [23]. Thus, the electrochemical studies complemented the spectroscopic results to propose the intercalative mode of binding between the terpenoid and DNA. The currents decrease dramatically in cathodic peak potential with increased anodic peak. It can be induced from this that the terpenoids had the ability to bind the DNA by intercalation mode [24].

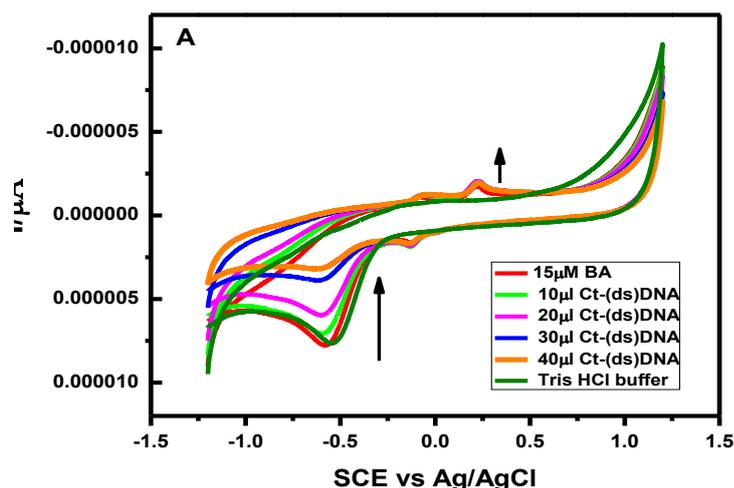


Figure: 6.A

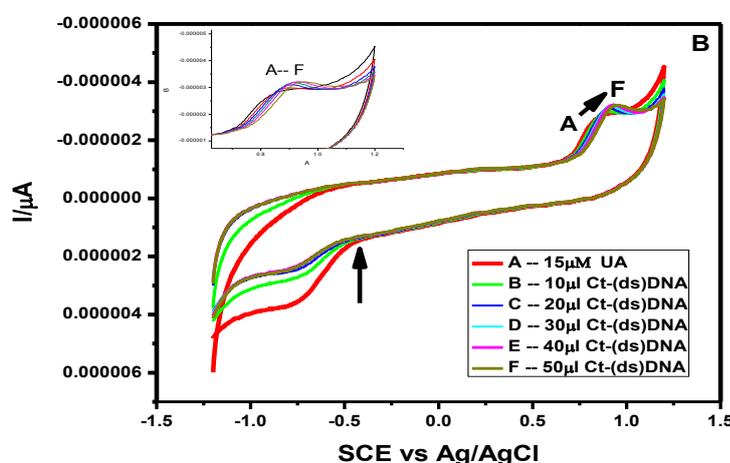


Figure: 6.B

Figure: 6.A & 6.B Cyclic voltammograms of interaction of (A)BA, (B)UA with various concentrations of Ct-(ds) DNA.

The binding constant was determined from the intercept of the plot of  $\log 1/[DNA]$  versus  $\log i_p / (i_o - i_p)$  using this equation (2)

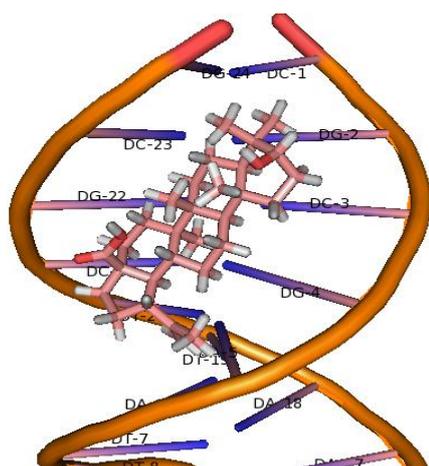
$$\log (1/ [DNA]) = \log K + \log i_p / (i_o - i_p) \quad \text{-----} > 2$$

Where K is the binding constant,  $i_o$  and  $i_p$  are the peak currents of the complex in the absence and presence of DNA, respectively. Typical cyclic voltammetric behavior s of terpenoids without and with Ct-(ds) DNA was studied in pH 7.4 Tris-HCl buffer solution under the potential range of (-1.2v to 1.2V) for both BA and UA with the scan rate of 50 mV s<sup>-1</sup> (Fig.6.A, B) and Oxidation & reduction peaks (redox reaction) appeared. As can be seen, on the bare GCE, terpenoids had a small stable and quasi-reversible redox response, however, on the GCE, much more obviously redox peaks of terpenoids were found at -0.5 and 0.25 V (BA) and -0.8 to 0.8(UA). Binding constant for Betulinic acid ( $2.158 \times 10^{-5}$ ) and Ursolic acid ( $3.2 \times 10^{-5}$ ).

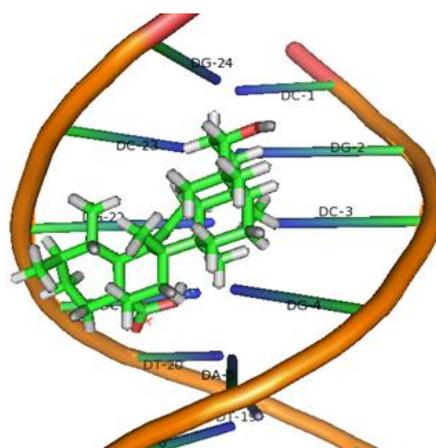
#### Molecular docking:

Molecular docking techniques are an attractive platform to understand the drug–DNA interactions in normal drug design, as well as in the programmed study by placing a small molecule into the binding site of the target particular region of the DNA mainly in a non-covalent fashion. In this paper, molecular docking studies were employed with Autodock (4.2) to know the interaction mechanism of terpenoids compounds binding with Ct-(ds) DNA along with the structural characteristics of an active site of the DNA <sup>[19]</sup>.

#### Visualization

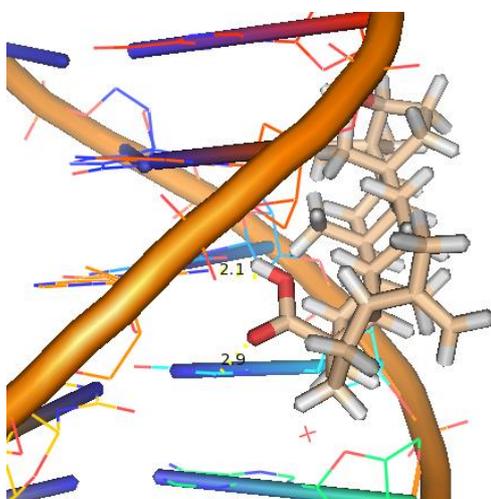


DNA with Betulinic Acid

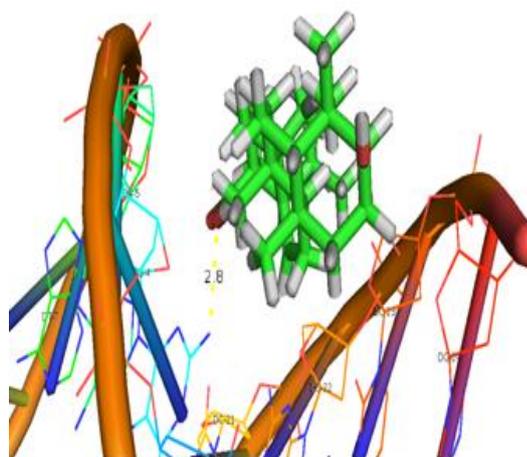


DNA with Ursolic Acid

**Distance measurement**

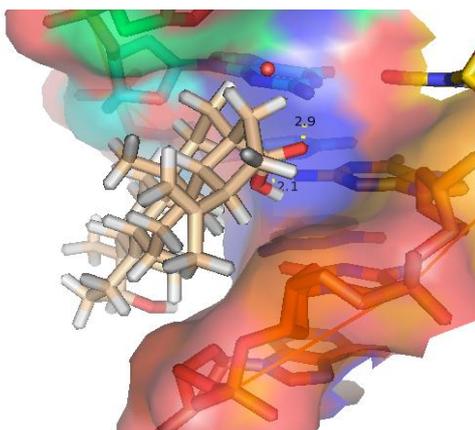


DNA with BA

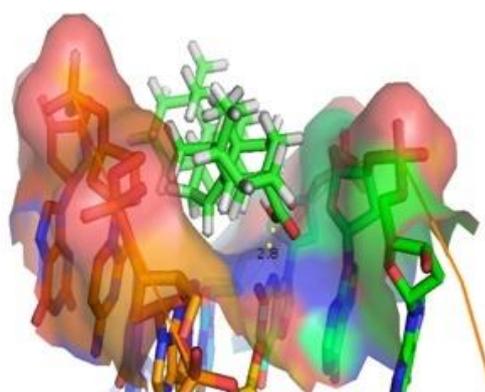


DNA with UA

**Surface transparent view**



DNA with BA



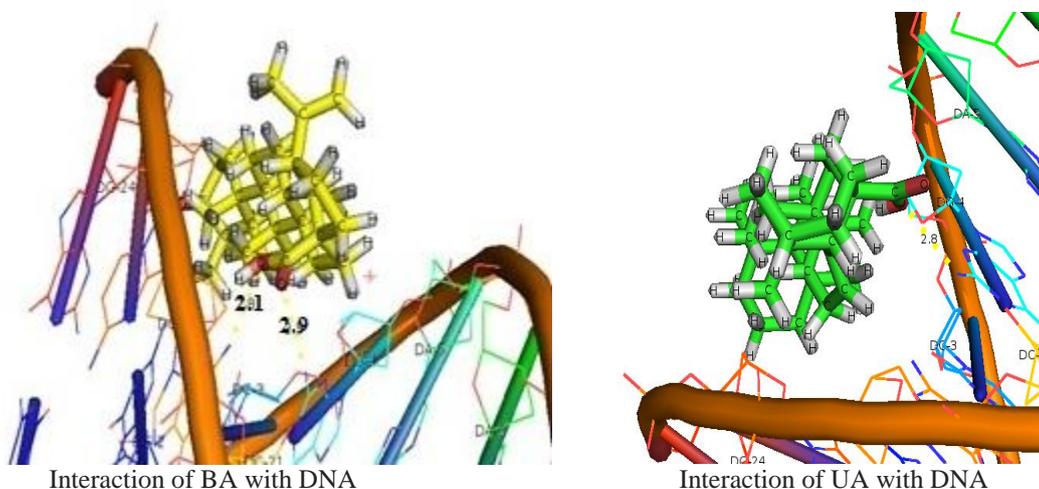
DNA with UA

**Hydrogen bonds and Binding Energy data obtained from molecular docking procedure of terpenoids with Ct-(ds) DNA**

No	Compounds	Interactions	Distance Å	Bonding	Bonding types	Binding site of ligand
1.	Betulinic Acid	DC3.....1BNA DG4.....1BNA	2.1 2.9	Hydrogen Hydrogen	H-bond H-bond	O O
2.	Ursolic Acid	DC3.....1BNA	2.8	Hydrogen	H-bond	O

Docking is a consummate model to explicate DNA interactions with pharmacologically active ligand leads to drug discovery and design. The active ligands were separately docked with DNA d (CGCGAATTCGCG) 2 (PDB ID: 1BNA).

**Interaction**



The binding interactions of ursolic acid and Betulinic acid Ct -DNA (1BNA) simulates to have intercalation mode of binding with DNA. In the midst of ligands, Betulinic acid has the features of more interaction potential than ursolic acid and they bound to a similar site of the DNA. Moreover, 10 poses were accomplished depending upon default parameters. Hydrogen bonding distances and their position particularly was at DG 4(2.8) for Ursolic acid DG 4(2.9) and DC 3(2.8) for Betulinic acid. Amidst two, the Betulinic acid shown a stronger binding efficiency compared to Ursolic acid.

**IV. Conclusion**

In summary, the binding interaction of terpenoids with Ct-(ds) DNA in Tris-HCl buffer was investigated by multispectroscopic techniques, electrochemical and molecular modeling studies. The results indicated that the binding mode of terpenoids to DNA is an intercalation binding, which was supported by the results from electrochemical studies. From the absorption spectroscopy, absorbance was increased and got the broad peak with the addition of various concentration of Ct-(ds) DNA, indicates that terpenoids were binding with Ct-(ds). Binding constant value of,  $K_b = 2.18 \times 10^{-5} \text{ mol}^{-1} \text{ L}$  (BA) and  $K_b = 3.16 \times 10^{-5} \text{ mol}^{-1} \text{ L}$  (UA). Furthermore, the DNA-binding ability of the complex was confirmed from Emission spectroscopy, which indicated that quenching process take place and interactions were clearly explained by molecular modelling approach. These studies may provide useful information for further study of the pharmacological effect such as inhibition of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anthelmintic, antinociceptive, anti-HSV-1, and anticancer activities.

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