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THE ANTIOXIDANT AND DNA DAMAGE PROTECTION ACTIVITY OF *Hibiscus sabdariffa* L.

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ABSTRACT

The extract of *Hibiscus sabdariffa* calyces are a rich source of healthy benefit phytochemicals, especially anthocyanins, which are flavonoid plant pigments with antioxidant effects. Roselle (Hibiscus) extract was prepared and the functional groups of the main compounds were detected by UV–VIS spectrum and Fourier transform infrared spectroscopy (FT-IR). The extract's antioxidant capacity was detected using radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging action and ferric reducing antioxidant power. The human genomic DNA damage inhibition was tested and *in - silico* studied by docking of some Roselles anthocyanin compounds into the DNA. The main functional groups of Roselle anthocyanin compounds were detected by FT-IR and their peaks appeared at 282 nm and 520 nm UV-VIS spectrum. Additionally, the Roselle extract shows a powerful reducing power activity and antioxidant activity with IC_{50} of about 105.9 ± 4.56 µg/ml besides the extract efficiently inhibits DNA damage by UV-radiant and free radicals. That was confirmed by the docking study of more common Hibiscus anthocyanin compounds, (Delphinidin 3 sambubioside) and (Cyanidin 3 sambubioside) into the DNA which formed docking conformations with stable bonds in the possible interaction of each of the compounds with the DNA as well as good binding energy (-5.62 and-6.74 kcal/mol) and lowest intermolecular energy (-10.69 and -11.24 kcal/mol) for them respectively. Therefore, Hibiscus calyces extract displayed potent human genomic DNA protection activity and can be used for DNA preservation and cancer inhibitors.

Keywords: *Hibiscus sabdariffa*; Roselle calyces; DNA damage inhibitor; Delphinidin 3-sambubioside; Cyanidin 3- sambubioside; DNA Autodock.

INTRODUCTION

For long years, humans have tried to find more beneficial foods or natural medicines that can delay, prevent, or cure the disease and keep their health in good condition. Most plant products like fruits, vegetables, and herbs form the best sources of nutrients that could achieve this goal because of their contents of macronutrients like carbohydrates, proteins, and fat as well as micronutrients like vitamins, minerals, and bioactive components (such as phytochemicals) that can avoid many diseases e.g. cardiovascular diseases and non-communicable diseases [1]. The tropical plant *Hibiscus sabdariffa* which belongs to the Malvaceae family is among the more vital plants in this field it's known under multiple names like Roselle, Sorrel, Kujarat, or karkade it's famous for its medicinal properties and flavor. It is one of the most widely used plants as food and medicine. The red petals (calyces) are already widely used as a food coloring and also used as tea is beneficial for the treatment of infections, cancer, cough, weakness, high blood pressure, and heart disease lowering blood cholesterol levels, lowering blood sugar levels for diabetics and detoxification (neutralizing poison) [1–3]. Aqueous extracts of Calyces used globally as antioxidant-containing foods and Roselle extract's polyphenolic compounds for instance flavonoids, phenolic acids, and anthocyanins which are remarkably reported to be the main sources of antioxidants [3,4], Anthocyanins are type flavonoid and water-soluble pigments that are produced by plants as secondary metabolites. Their glycons are named anthocyanidins [5]. They are flavorless and odorless and vary in kind from purple, blue, red, orange, or in the coloration of different plant organs such as tubers, fruit, root, leaf, stem and flower [6–9]. The stability of anthocyanin is dependent on temperature, structure, light, and pH [10].

The high consumption of foods rich in anthocyanin provides beneficial health properties for various illnesses accompanying bacterial infections, inflammation cancer, aging diseases, neurological diseases, obesity and diabetes besides they play a significant treatment role for liver disorders, diarrhea, hypertension, the common cold, urinary tract infections, and stones of the kidneys [5,10]. In folk medicine, Anthocyanin pigments have been used throughout the world. For instance, anthocyanins of bilberry have long been used for vision complaints, microbial infections, and diarrhea treatment [11].

Anthocyanins have been shown to work as a "sunscreen" guarding cells from damage by UV light absorbing, additional to their ability to prevent the generation of free radicals by blue light and UV by their effective antioxidant property [9]. The current study aim is to detect the antioxidant features of the red petal (calyces) extract of Roselle and study its ability to conserve human DNA from damage by UV and free radicals.

MATERIALS AND METHODS

Chemicals and Reagents

The chemicals used in the study were $FeCl_3$, (K3 Fe (CN)) potassium ferricyanide, (DPPH) (2,2-diphenyl-1-picrylhydrazyl), Ascorbic acid (AS) and all the additional chemicals and solvents used were supplied from the laboratories of Pharmacy college in the University of Basra.

Preparation of Extract

The extraction method was done as stated by Mohamed et al. [12], a ten-gram of dried grounded Roselle calyces - collected from the local market in Basra- were mixed with 100 ml of the extracting solution (ethanol and (1% HCl) acidified water 1:1) in an Erlenmeyer flask covered with Aluminum foil and were stirred with a magnetic stirrer at room temperature for 6 hours then stand in the similar condition for 24 hours. Anthocyanin extracts are then filtered by Whitman's number one filter paper to remove fibrous particles. The filtrates of extracting medium were dried in the dark at room temperature then the dried extract collect in a dark container and kept at -20°C.

Estimation of Hibiscus Extract's Antioxidant Action

Radical (DPPH) scavenging capacity

The Hibiscus calyces extract antioxidant action was measured as a radical scavenging capacity of

DPPH. The Erenler et al. [13] method with a little modification was followed to accomplish the experiment. The absorbance readings at 517 nm were decreased with the reduction of the radicals. 0.5 ml of 0.2 mM (DPPH) methanolic solution was added to 2.5 ml of each concentration of Hibiscus aqueous methanolic extract (12.5 - 200) µg/ml. The tubes were enclosed tightly and set aside for (0.5) hours in the dark then at 517 nm the absorbance against blank samples was measured and compared to the calibration curve of ascorbic acid. The test was accomplished in triplicate. The radical % inhibition was gained for the (DPPH) by the below equation:

$$I\ \% = \frac{A0 - A}{A0} \times 100$$

where I = inhibition of DPPH (%), A0 = control sample absorbance and A = tested sample absorbance after 0.5 hour.

The scavenging activity plotted graph against different *Hibiscus sabdariffa* extract concentrations can be used for determining the IC50 value, which can be defined as the total antioxidant essential to decrease 50% of the initial radical (DPPH) concentration. reference compound was the Ascorbic acid [14].

Estimation of Antioxidant Ferric Reducing Power (AFRP)

Determination of antioxidant reducing power was evaluated by Vijayalakshmi and Ruckmani method [15], that different concentration of Hibiscus extract (5-50 µg/ml) was added to (2.5 ml) of (pH 6.6) sodium phosphate buffer 200 mM plus (2.5 ml) of 1% solution of [K3Fe(CN)6] potassium ferricyanide. Then the mixture was well vortexed and then the latter at 50°C were incubated for 20 minutes, after that 10% trichloroacetic acid (2.5 ml) was added to the reaction mixture and centrifuged for ten minutes at 3,000 rpm. The deionized water (2.5 ml) with (0.5 ml) of ferric chloride 0.1% was mixed with (2.5 ml) of the supernatant. The absorbance at 700 nm for the colored solution against the blank was measured by UV Spectrophotometer with ascorbic acid as a reference standard. The samples reducing power was compared with the reference standard.

Identification of Some Compounds in Hibiscus Extract by FT-IR and UV-VIS

Many of the literature on *Hibiscus sabdariffa* revealed that the aqueous extract of Hibiscus contains multiple compounds, especially anthocyanins such as (Cyanidin-3-sambubioside) and (Delphinidin-3-sambubioside), which were identified as the extract major constituent [11,16,17,18]. Therefore, in this study, the red extract of Roselle calyces obtained by (aqueous solution with1% HCl and ethanol as solvent) is analyzed by spectrophotometer UV-VIS. Also, the Roselle calyces extract undergo IR detection using Shimadzu 8201 PC to recognize the main active chemical groups.

***In vitro* Study the Effect of *Hibiscus sabdariffa* Calyces Extract on Genomic DNA**

The test was done by the following steps

Extraction of DNA

A human genomic DNA from human WBC was extracted by using the Geneaid DNA extraction kit and all the extraction steps were done according to instructions of the kit supplied company.

The effectiveness of the DNA damage inhibition

The Roselle extract's ability test for DNA protection was achieved according to Russo et al. with a few modifications, using human genomic DNA, through the presence of H_2O_2 and UV radiation and the electrophoresis of agarose gel performance for the (irradiated) DNA [19-21]. 5 µl aliquot of human genomic DNA (20 µg/ ml) was added to 5 µl of each of two concentrations of Roselle calyces extract (2 and 1) mg/ml in a microcentrifuge tube. Also, another tube contains all the contents but the plant extract is called irradiated control (IC). After that, we added 5 µl of H_2O_2 (3%) to all tubes (including IC). Finally, tubes were exposed to (300 nm) of UV transilluminator at room temperature for 10 min. An individual tube contains 1 µl aliquot of human genomic DNA was placed also, which was implemented as the non-irradiated control. The agarose gel (1%) electrophoresis was used for

running all the samples then the DNA bands were photographed.

Molecular Docking of Some of Hibiscus Calyces Extract Compounds into the DNA

The molecular docking for some anthocyanin compounds of Roselle calyces extract into the DNA was performed by AutoDock 4.2 software program 1.5.6 which is offered free below the Public License of (http://AutoDock.scripps.edu/) (GNU General) and employed for molecular autodocking and binding scoring by the following steps:

Preparation of both the DNA and ligands

The download of DNA strand three-dimensional structure was done from Protein Data Bank under PDB ID 195D [22], the properties of the DNA was reported in Table 1, before the docking simulations. All water molecules, ions, and ligands were removed and hydrogen ions were added.

And Fig. 1 reports the chemical structures of the major anthocyanin compounds found in the Hibiscus calyces extract as mentioned by Saranraj and Devi [16]. The (3D) 3-dimensional structures of Roselle anthocyanin compounds were downloaded from PubChem in SDF format. https://pubchem.ncbi.nlm.nih.gov/.

Docking and building complexes

The docking procedures by AutoDock consisted of the following: First, detect the selected DNA binding sites and ligand conformation of the sample in it by using the Lamarkian Genetic Algorithm, depends on the energy grids which calculated previously. While the binding site can be distinct as the atoms within 6Å of the cognate ligands, 2,500,000 was the number of energy assessments for each docking run and 0.375Å was set to the grid spacing. Second, the binding scores for diverse conformations were consequently determined by the AutoDock scoring function [23,24].

Table 1. The human DNA crystallographic features

DNA	PDB code	Classification	Organism	Method	Total structure weight (DA)	Chain
DNA duplex	195D	DNA	-	X-RAY diffraction	7760	A, B

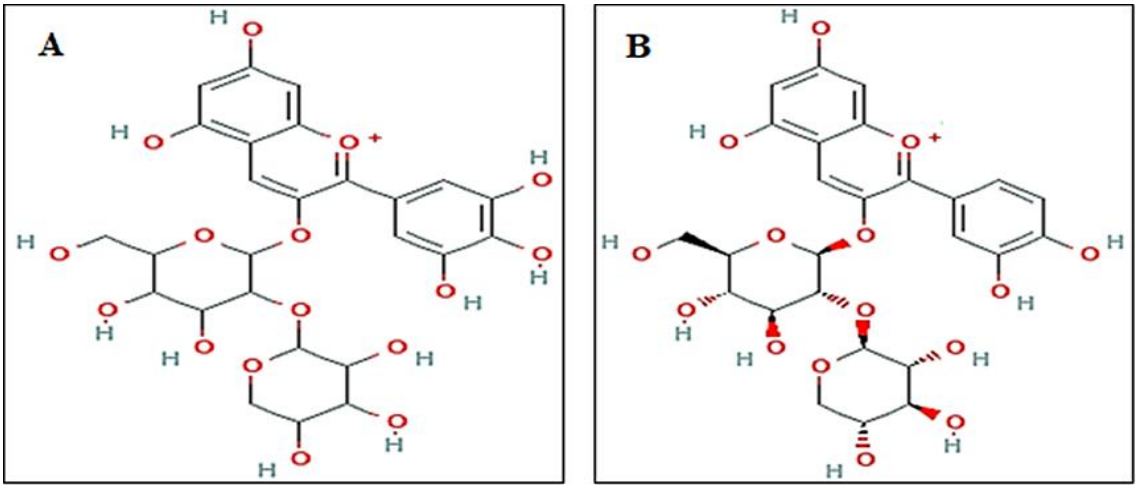

Fig. 1. The selected anthocyanin compounds were found in Hibiscus calyces extract. A. Delphinidin-3-sambubioside, and B. Cyanidin-3-sambubioside which were gathered from the Pub Chem site

RESULTS

The Yield of Extraction

Yields of Roselle calyces crud extract by using ethanol with acidified water solution was 0.72 g/ 100 g.

Antioxidant Activity

In the study, the *Hibiscus sabdariffa* antioxidant capacity for extract was designated in Fig. 2. Which showed the radical scavenging action of (DPPH) over multiple Hibiscus extract concentrations (12.5-200) µg/ml and the IC50 of extract's DPPH scavenging activities was 105.9 ± 4.56 µg/ml.

Fig. 3 depicts antioxidant ferric reducing power (AFRP) for multiple *Hibiscus sabdariffa* extract's concentrations, the extract's reducing power was raised with the concentration increase. The reducing power ability was compared with standard Ascorbic acid at 5-50 µg/ml concentration at 0.11- 0.038 nm for O.D respectively.

Identification of the Roselle Calyces Extract Compounds by FT-IR

The anthocyanin are Polyphenolic compounds that absorb infrared radiation, and the interpretation of infrared absorption bands of anthocyanin's aglycone is more easily observed in the region 1800-1380 cm^{-1}. As shown in Fig. 4 the absorption bands at other areas also used to support the anthocyanin's existence in (*Hibiscus sabdariffa*) extract, for example from FT-IR spectrum, there are two broad absorptions at 3417.86 and 3159.4 cm^{-1} which is the characteristic absorption band of hydrogen bonding (-OH). While there is 3 aromatic C-H stretch absorption at 3086.11, 3047.53, and 3005.1 cm^{-1}. Absorption at 1743.65 cm^{-1} is distinguishing for five-member cyclic -C=O- stretching. The emergence of absorption at 2320 cm^{-1} indicates the presence of —C═O⁺—C— which conjugated to the aromatic ring and supported by absorption at 1276.88 and 1261.45 cm^{-1} from outreach the symmetric =C-O-C the C-O stretch of aromatic alcohol indicated at the absorption of 1215 and 1188.5 and 1072.42 cm^{-1}. With these data, it can be ascertained the presence of the polyphenolic compounds of anthocyanins.

Identification of Anthocyanin Compounds by UV-VIS Spectroscopy

The UV-VIS spectrum (Fig. 5.) of *Hibiscus sabdariffa* extracts illustrated some peaks at multiple wavelengths, UV λ max 282 nm, 520 nm and 546 nm.

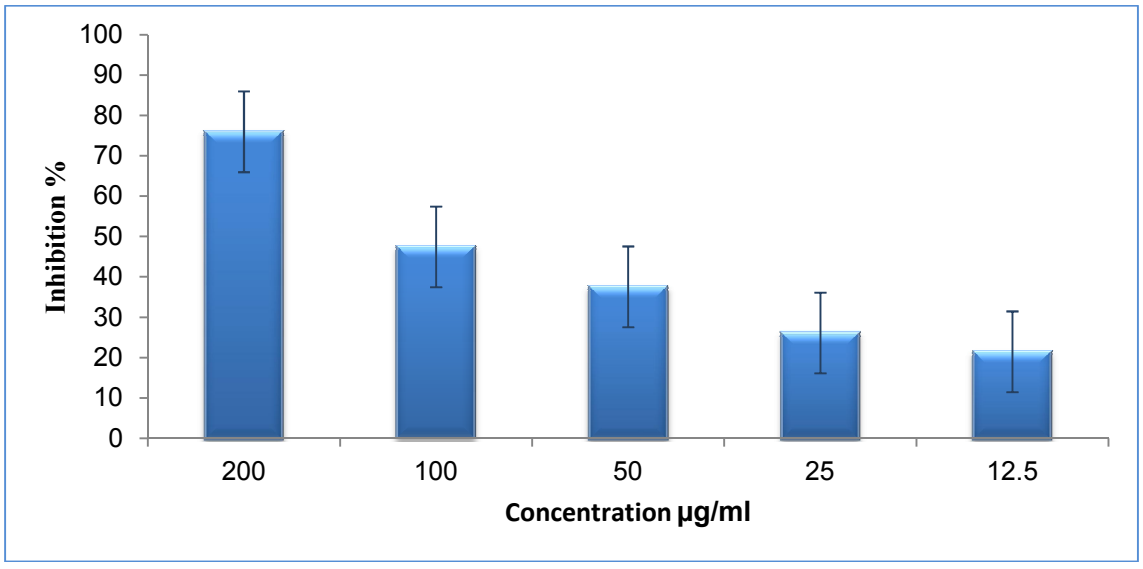

Fig. 2. The DPPH inhibition% for each concentration of Roselle calyces extract

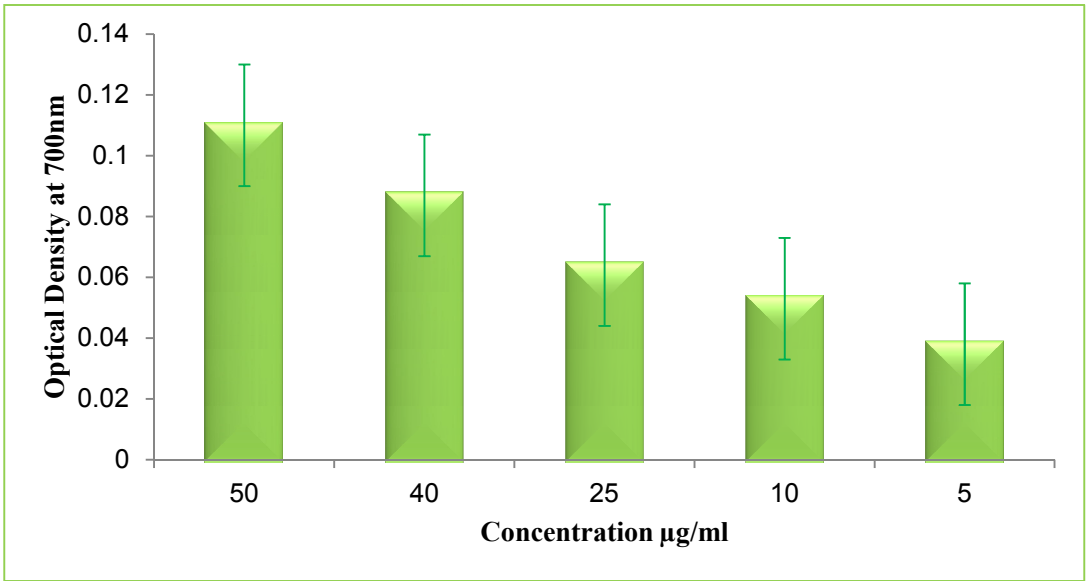

Fig. 3. Antioxidant ferric reducing power of Hibiscus calyces extract

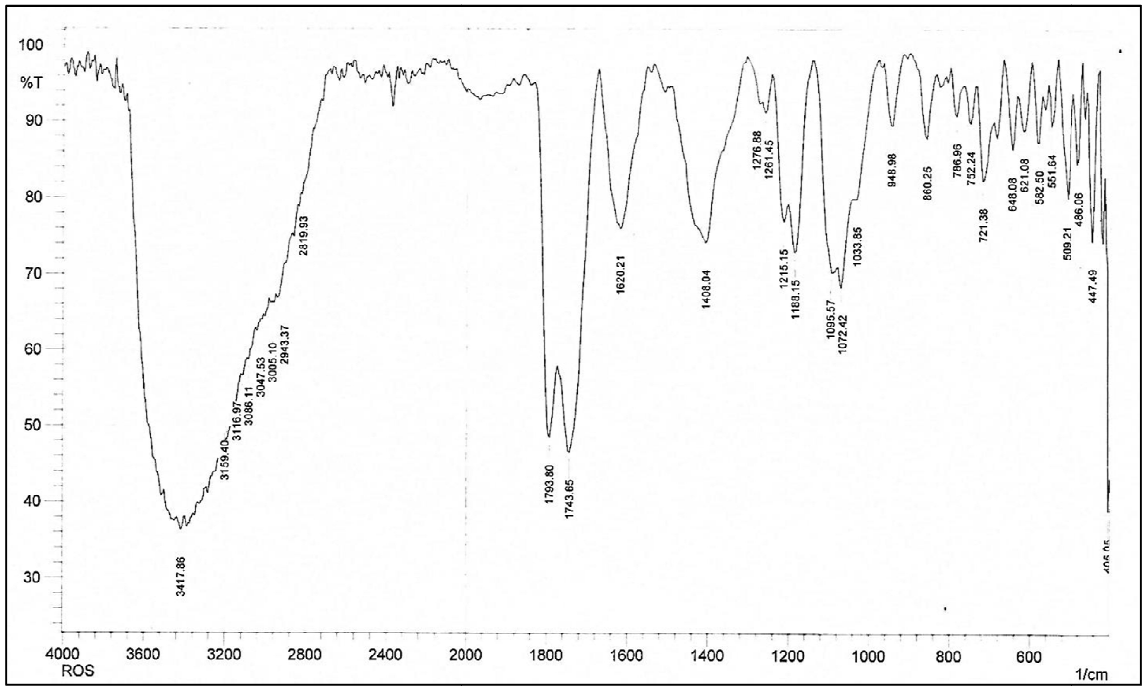

Fig. 4. The FT-IR of some of the Roselle calyces extracts compounds

The Efficiency of Inhibition the DNA Damage

Results showed that the extract at a dose of 1 and 2 mg/mL was exhibited completed protection of human genomic DNA. As shown in Fig. 6. In the figure, we can see that in lane 1 and 2 the human genomic DNA is protected from the damaging effect of facing UV radiant and highly oxidative

hydrogen peroxide H_2O_2. While in lane 3 the DNA form a smear (due to damage of DNA band by the effect of UV plus H_2O_2) as compared with the DNA band of (DNA without any addition or treatment) in lane 4.

Molecular Docking of Some Compounds of Roselle Calyces Extract into the DNA

The two Anthocyanin compounds (ligands) were docked into the DNA oligonucleotide $(CGCGTTAACGCG)_2$ and the ten poses of docking for each of the Anthocyanin compounds were collected within a Root-mean-square deviation (RMSD-tolerance of 2.0). The AutoDock- conformations that form hydrogen bonds mostly resemble the hydrogen bonding mode of the DNA in the X-ray crystallographic conformation was chosen as shown in (Figs. 7, 8 and 9) and Table 2 shows the more important docking energy scores, the binding free energy, electrostatic and intermolecular energy addition to cluster RMSD for each of the docking conformations.

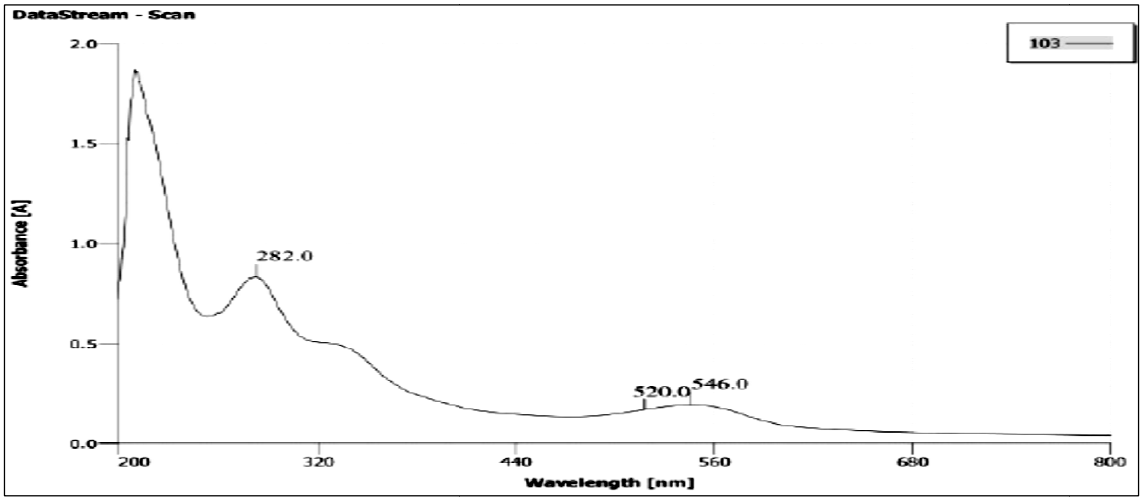

Fig. 5. The UV-VIS spectrum of anthocyanin compounds in Roselle calyces extract

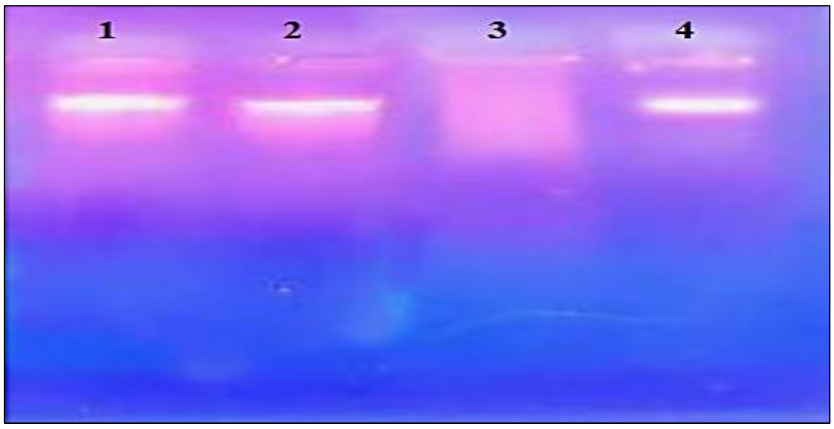

Fig. 6. The efficiency of inhibition of the DNA damage. Lane 1 and 2 represent the two concentrations of (1 and 2 mg/mL) of Roselle calyces extract with human DNA and H_2O_2 faced the UV. While lane 3 represents the irradiated control (IC) (the human DNA and 3% H_2O_2 with UV) and the nonirradiated control DNA in lane 4

Table 2. The important docking energy scores and cluster RMSD for the chosen Autodock-conformations

Anthocyanin compounds	Binding energy *Kcal/mol	Intermolecular energy Kcal/mol	Electrostatic energy Kcal/mol	Cluster RMSD
Delphinidin3-sambubioside.	-5.62	-10.69	-0.28	zero
Cyanidin3- sambubioside.	-6.74	-11.24	-0.94	zero

*Kcal: kilocalorie

The possible interactions of the DNA with Delphinidin 3-sambubioside and Cyanidin3-sambubioside, as well as the types of bonds, distances, and energy of each bond were shown in Table 3.

DISCUSSION

Recently there has been increasing interest in Roselle (*Hibiscus sabdariffa*) which may be due to their beneficial health effects especially the antioxidant and anticancer activity that is mostly related to the presence of Anthocyanin compounds, and naturally, the *Hibiscus sabdariffa* calyces regard as rich anthocyanin source [25], where our study tries to prepare crude Roselle calyces extract in a special manner targeting the anthocyanin compounds as recommended By Mohamed et al. [12]. Fifty years ago Blois introduced the Diphenylpicrylhydrazine (DPPH) method, DPPH is a nitrogen stable center of free radical used to assess the plant extract's scavenging ability for the free radicals, as well as to estimate antioxidant capability. The DPPH acceptance of an electron from the antioxidant compound (reducing factor) turned the violet radical (DPPH) solution to a yellow one, the DPPH can be colorimetrically measured [14,26]. Compounds that reduced the DPPH can be considered as radical scavengers and therefore antioxidants [27,28]. The Roselle extract shows strong free radical scavenging activity comparing with standard vitamin C which gave 21% inhibition for the free radical at 200 µg/mL and that made *Hibiscus sabdariffa* extract a powerful antioxidant product as mentioned by multiple studies like [29–31].

Additionally, the flavonoids, as well as phenolic acids can also reveal another antioxidant mechanism by donating an electron to Fe (III) and reduce it to Fe (II), and that is called compounds reducing power that normally based on the reductones, the latter break the chain of the free

Table 3. The bonds of the possible interaction of DNA with Anthocyanin compounds

(Ligands) Anthocyanin compounds	DNA interaction molecules		DNA chain	Hydrogen bonds type	Distance (A^0)	Energy (kcal/mol)
1. Delphinidin3-sambubioside.						
O49	O3`	DT5	A	H-donor	2.85	-2.3
O41	O2	DC3	A	H-donor	3.41	-1.1
O45	OP1	DC23	B	H-donor	2.64	-0.7
O20	C1`	DT 5	A	H-acceptor	2.78	-0.7
O14	C5`	DG 22	B	H-acceptor	3.37	-0.5
O16	C4`	DC 21	B	H-acceptor	2.89	-0.7
2. Cyanidin3- sambubioside.						
C1	OP1	DC 11	A	H-donor	3.6	-0.7
C3	OP1	DC11	A	H-donor	3.2	-1.9
C49	OP1	DG 10	A	H-donor	2.99	-1.3
O16	OP1	DT 18	B	H-donor	2.76	-0.9
O18	OP1	DT 18	B	H-donor	2.63	-3.3
O20	O4`	DA 19	B	H-donor	2.69	-2.4
O45	OP1	DC 11	A	H-donor	3.65	-0.7

DC: Deoxycytosine, DG: Deoxyguanosine, DT: Deoxythiamine, DA: Deoxyadenosine, O: Oxygen atom, P: Phosphate atom, C: Carbon atom

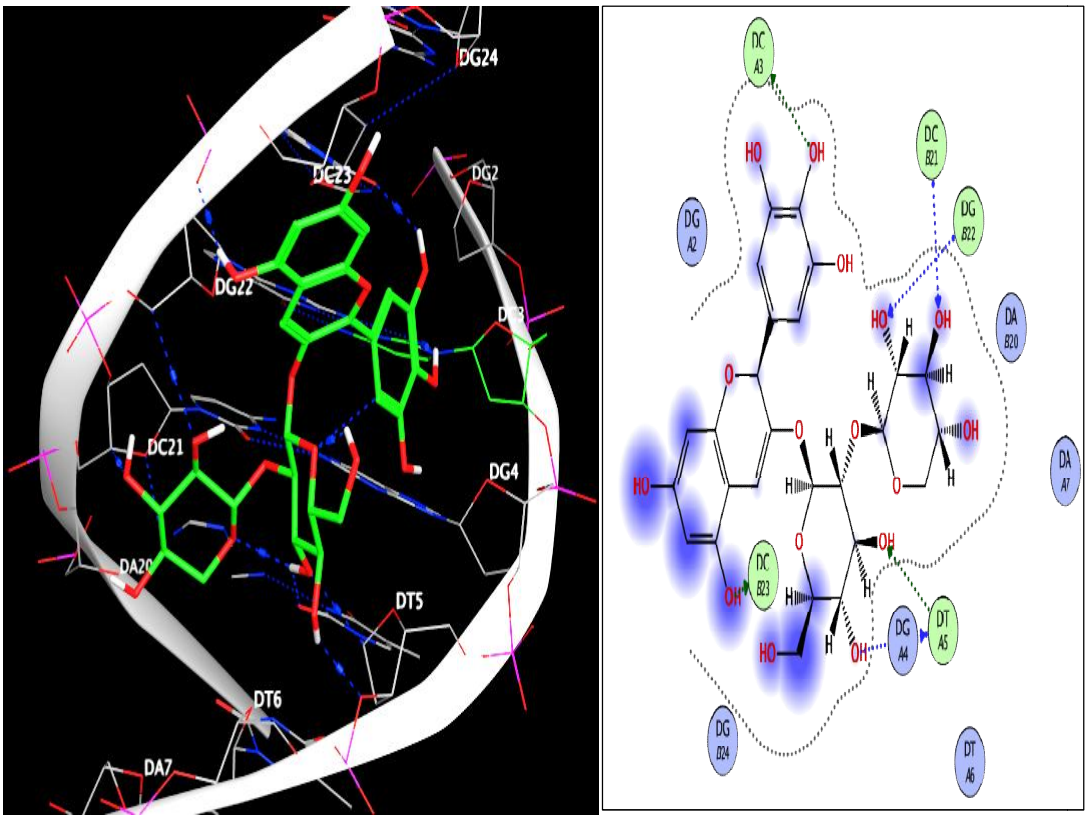

Fig. 7. The 3D and 2D of the possible interaction of Delphinidin 3-sambubioside with the DNA

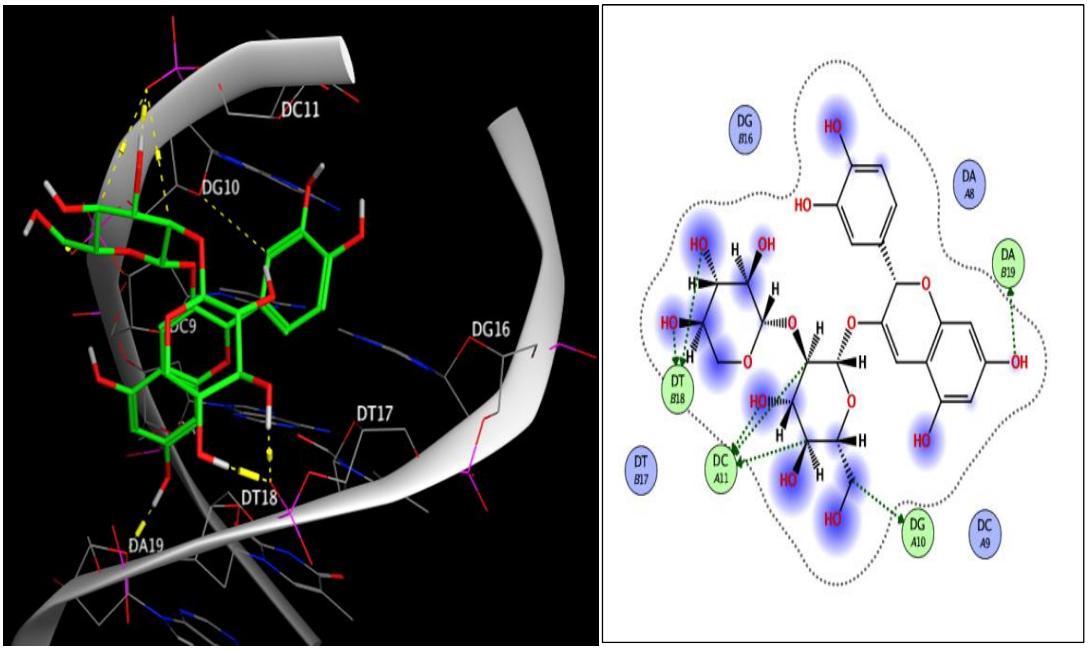

Fig. 8. The 2D and 3D of the possible interaction of Cyanidin 3- sambubioside with the DNA

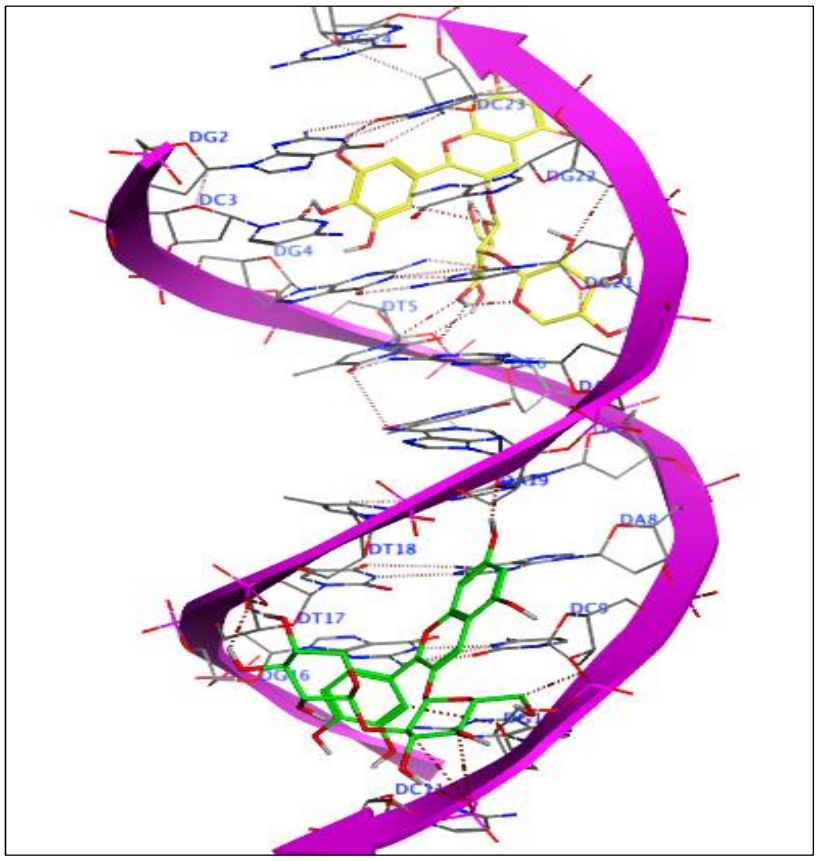

Fig. 9. The 3D possible interaction of DNA with both anthocyanin compounds (yellow) Delphinidin 3-sambubioside and (green) Cyanidin 3- sambubioside

radical by donating hydrogen ion and display antioxidant activity. This technique uniquely distinguishes the electron donator existing in the extracts. The Fig. 3 showed obviously a high antioxidant potential (reducing power ability) for the Roselle extract in comparable with the standard vitamin C in the same concentration and the ability of formation of the ferrous compounds by reducing the ferric ones and which was increased with an increase of extract concentration [32–34]. The UV-VIS spectrum for the Roselle extract show multiple peaks at wavelength 282 nm and 520 nm and that wavelength specific for aqueous extracted anthocyanin from *Hibiscus sabdariffa* and that was reported in earlier studies [35–37]. While the peak at 546 nm may be related to the using of ethanol alcohol as a solvent during evaluating the UV-VIS spectrum which can lead to a shift in UV absorption for some of these compounds [38,39]. Additionally, the more important anthocyanin in *Hibiscus sabdariffa* extract is cyanidin-3-sambubioside, delphinidin-3-sambubioside, cyanidin-3-glucoside, and delphinidin-3-glucoside. Each of these molecules may work as a copigment molecule for the other anthocyanin compounds and form what is called intermolecular copigmentation which is defined as the modification in UV-visible absorption of anthocyanins when they are located close to another anthocyanin compound the latter is called copigment and lead to modified or shift the UV-visible absorption of anthocyanins as shown in Fig. 5 [40].

Infrared (IR) or Fourier transform infrared (FT-IR) spectroscopy means measures the molecule's absorption of electromagnetic radiation, when molecules absorb infrared radiation of a certain

energy, their bonds vibrate and one can measure the amount of absorbed energy in each specific wavelength and make interpretations about the bonds types for each molecule [41]. The spectrum for Roselle extract FT-IR shows a characteristic infrared absorption band for the functional groups of the polyphenolic compounds of anthocyanins [2].

In this study, the DNA oxidative damage protective potentials were detected by using UV radiation plus H_2O_2, the later often used as an experimental source of free radicals derived by oxygen and its significant tool for studying their effect on DNA that may cause a break of single and double DNA strands in some human cells [42]. Whereas Ultraviolet radiation mainly from wavelength 280 to 315 nm regarded as one of the causes for the alteration of ordinary life state by promoting a diversity of cytotoxic and mutagenic lesions of DNA e.g 6-4 photoproducts and cyclobutane-pyrimidine dimers in addition to interfering with the integrity of the genome by breaking DNA strand [43]. Otherwise, UV‑photolysis of H_2O_2 generates OH radicals, which is responsible for most of DNA and protein oxidative destruction, also DNA binding with the OH radical leads to deoxyribose sugar destruction, and modification of the nitrogen base [21]. The current study took up, the anthocyanin compounds of *Hibiscus sabdariffa* extract which mainly has strong antioxidant action shows an extensive protective activity for the human genomic DNA against free radicals that mediated DNA damage and therefore, can be used in cancer inhibition or at least for DNA protection. To examine the role of Roselle extract in DNA protection, some of the anthocyanin compounds for instance delphinidin-3-sambubioside and cyanidin-3-sambubioside which are majorly found in *Hibiscus sabdariffa* extract was docked into the DNA [35]. The Molecular docking is considered as the best applicable approach for the theoretical understanding of the mechanism of molecular interaction and for explanation the binding types of the DNA with the compounds through the noncovalent interactions [44]. Both anthocyanin compounds display good binding energy and forming stable hydrogen bonds with the lowest intermolecular energy, the latter bonds with DNA work to inhibit the OH radicals actions.

CONCLUSION

The *Hibiscus sabdariffa* calyces extract which contains anthocyanin compounds proved to have high antioxidant activity and Ferric reducing power additionally they displayed a powerful human genomic DNA protection activity against the free radicals and UV-radiation. Therefore these compounds can be used for DNA preservation and cancer inhibitors and the study of the molecular docking for some of Hibiscus anthocyanin compounds with DNA confirmed their role in DNA protection activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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