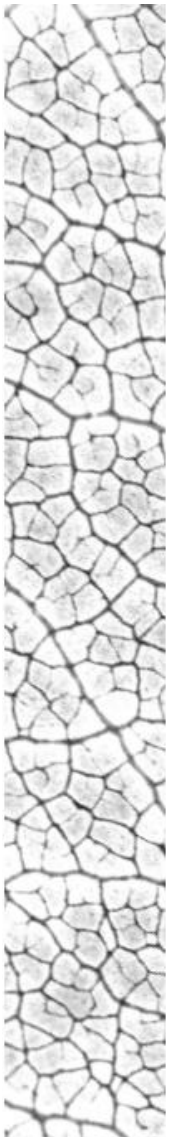

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Estimate the total flavonoids content, antioxidant activity, and DNA damage protection for the methanolic extract of Stachys sp. gathering from the Basra market in Iraq

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Abstract
The expansion of cancer disease, cardiovascular disease, diabetes, and recently COVID-19 have guided scientists toward the utilization of natural products from plants because of cheap and availability. Flavonoids one of the most important compounds extracted from the plant such as genus Stachys in this research, which illustrate that the MtOH extract of Stachys sp. has 103.36 mg of flavonoids content and also the result of scavenging ability of extract on hydrogen peroxide show the highest inhibition percentage (38%) belongs to (2 mg/ml) of extract. The result of reducing power activity illustrates high reducing activity for concentrations (10-50 µg/ml). DNA damage protection experiment showed the ability of Stachys sp. MtOH extract to protect human genomic DNA at two concentrations (2 and 1 mg/ml). Molecular docking using AutoDock 4.2 software was used to explain the binding mode between two selected flavonoids (apigenin and luteolin) in Stachy extract to help us understand the ability of flavonoids for DNA protection, results illustrate the best conformation with free binding energy (-6.86 & -7.11) for apigenin and luteolin, respectively. The results also show the formation of hydrogen bonds with the DNA (1N37) double helix.

Keywords: flavonoids, Stachys sp., apigenin, luteolin, antioxidant, DNA protection

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INTRODUCTION

The genus Stachys locally is known as “Butnig” in Iraq, it’s classified under Lamiaceae and contain around 300 species worldwide, this genus is concentrated in the warm temperate regions of the Mediterranean and southwest Asia, with secondary centers in North and South America and Southern Africa (Erdoğan, 2012). Many Stachys species are used in decoctions or infusions for the treatment of skin, stomach, ulcer, asthma, rheumatic disorders, and vaginal tumors. Some members of the genus have been reported to be used as anti-inflammatory and antibacterial agents. Moreover, their antianxiety, antioxidant, and antinephritic properties have also been reported. In Mediterranean regions and Iran, the species are consumed as herbal remedies and wild tea (mountain tea), in Iraq Stachys use as a food flavor and as a traditional remedy for gastrointestinal disorders (Gören, 2014). The uses of Stachys species in traditional medicine came from the bioactive compound in this genus such as essential oil, flavonoids, and phenolic acids, Similar to many other representatives of the family Lamiaceae, Stachys species produce essential oils, essential oils are complex mixtures and despite the large size of the genus Stachys, the composition of the volatile compounds is known in only a small number of species. As most of the plants belonging to the Lamiaceae family, the Stachys taxa have been submitted to several investigations to determine the content of the biologically active compounds, these investigations have reported the presence of flavonoids, phenolic acids, iridoids, and terpenoids (Vundać, 2019). Flavonoids are the most well-known phenolic compounds with strong antioxidant properties, the protective effects of flavonoids in biological systems have been attributed to their antioxidant capacity, disposal of free radicals, activation of antioxidant enzymes, and reduction of alpha-tocopherol radicals, flavonoids can prevent platelet accumulation and have anti-inflammatory, antibacterial, and antitumor properties (Elahe et al., 2016; Armutcu et al., 2018). In nature, flavonoid compounds are extracted from plants and found in several parts of the plant (Hilali

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et al., 2020). Also, they are used by vegetables for their growth and defense against plaques.

Many flavonoids are easily recognized as flower pigments in most angiosperm families. However, their occurrence is not restricted to flowers but are found in all parts of plants. They are also abundantly found in foods and beverages of plant origin, such as fruits, vegetables, tea, and cocoa (Panche, Diwan and Chandra, 2016). Today, phytotherapy, as the use of plant-based products or herbal extracts, is a common approach worldwide. Due to the low cost and availability of medicinal plant in the market the uses of medicinal plant is suitable as supplements or alternatives to synthetic drugs. In this study, we use Stachys sp from the Basra market which is used in many traditional remedies to estimate the total flavonoids content and antioxidant activity.

MATERIALS AND METHODS

Plant collection

Plant dried leaves were collected from the Iraqi local market then we grind it to a fine powder by the electric mill.

Preparation of plant extract

Plant powder was extracted by stirring of 10g of leaves powder with 100ml of 70% methanol for 5h, the extract was filtered by using Whatman number 4 filter paper. Then freed of solvent by evaporation at room temperature, the dried crude extract was stored at -20C°(Sytar et al., 2018).

Tests for flavonoids

The following tests were used for the detection of flavonoids in Stachys sp.

a-Shinoda test: To a methanolic solution of extract a few fragments of magnesium ribbon and concentrated hydrochloric acid were added. Appearances of red to pink color after a few minutes indicated the presence of flavonoids.

b-Ferric chloride test: few drops of neutral ferric chloride solution were added to little quantity of ethanolic extract. The formation of blackish-green color indicated the presence of flavonoids.

c-Lead acetate test: to the methanolic extract, few drops of aqueous basic lead acetate solution were added. The formation of yellow precipitate indicated the presence of flavonoids (Makhawi and Hamadnalla, 2019).

Determination of flavonoids content

Total flavonoid content was determined by the aluminum chloride method. 0.5 ml of the methanolic extract mixed with 0.3 ml of 5% sodium nitrite. After 5 min 0.3 ml of 10% aluminum chloride was added. After 6 min, we add 2.0 ml of 1 M sodium hydroxide and the total volume was made up to 5.0 ml with distilled water. The absorbance of the mixture was measured at 510 nm against a reagent blank. We use Quercetin as a standard. The flavonoid content was expressed as milligram of quercetin equivalence (QE) per gram of extract (Sasikumar, 2014).

Hydrogen peroxide scavenging assay

The ability of Stachys sp. plant extracts to scavenge hydrogen peroxide was determined by the prepared solution of hydrogen peroxide (43 mM) in phosphate buffer (1 M pH 7.4). We add Different concentration of sample (2-0.5 mg/ml) to a hydrogen peroxide solution (0.6 ml, 43 mM). The absorbance of hydrogen peroxide at 230 nm was estimated after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. We use ascorbic acid as a standard. Finally, we determined free radical scavenging by evaluating % inhibition (Sasikumar, 2014).

% inhibition = [(Control- Test)/control]×100

Reducing power assay

To evaluate reducing power activity we add to different concentrations of methanolic extract of Stachys sp (10-50 µg/ml) 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K3Fe(CN)6] solution. After that, the reaction mixture was vortexed well and then incubated at 50°C for 20 min using a vortex shaker. At the end of the incubation, we add 2.5 mL of 10% trichloroacetic acid to the mixture and centrifuged at 3,000 rpm for 10 min. (2.5 mL) from the supernatant was collected mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride, we determined the absorbance at 700nm against the blank. We determined the reducing power of the samples in comparison with the reference standard, and we used ascorbic acid as the reference standard (Vijayalakshmi and Ruckmani, 2016).

DNA damage protection assay

DNA damage assay was evaluated by using human genomic DNA from WBC. To extract DNA, the Geneaid DNA extraction kit was used, and we followed all the extraction steps according to the instructions supplied by the company.The protective capacity of the MtOH extract of Stachys sp.was achieved according to (Russo et al., 2001) method with modification. Using photolyzing human genomic DNA, through UV radiation in the presence of H2O2 and the performance of agarose gel electrophoresis with the irradiated DNA. In two polyethylene microcentrifuge tubes, 5 µL aliquots of human genomic DNA (20 µg/ml) were added, followed by two concentrations of Stachys sp. Extract (2 and 1 mg/ml). Another tube was irradiated control (CR) which did not contain extract. Then, 5 µL of 3% H2O2 was added to each tube. Next, they were placed on the surface of a UV transilluminator (300 nm) for 10 min at room temperature, directly. In another tube, 1 µL aliquot genomic DNA was placed and served as a non-irradiated control (CO). All samples were run on 1% agarose gel and then photographed (Bekhouche et al., 2018).

Table 1. Lipinski's physicochemical parameter for selected flavonoids (Apigenin&Luteolin)

Selected flavonoids	Molecular weight	h_logP	h_logS	a_acc	a_don	b_rotN	TPSA	lip_druglike
Apigenin	270.2400	2.6393	-3.4924	4.0000	3.0000	1.0000	86.9900	1.0000
Luteolin	286.2390	2.1557	-3.2180	5.0000	4.0000	1.0000	107.2200	1.0000

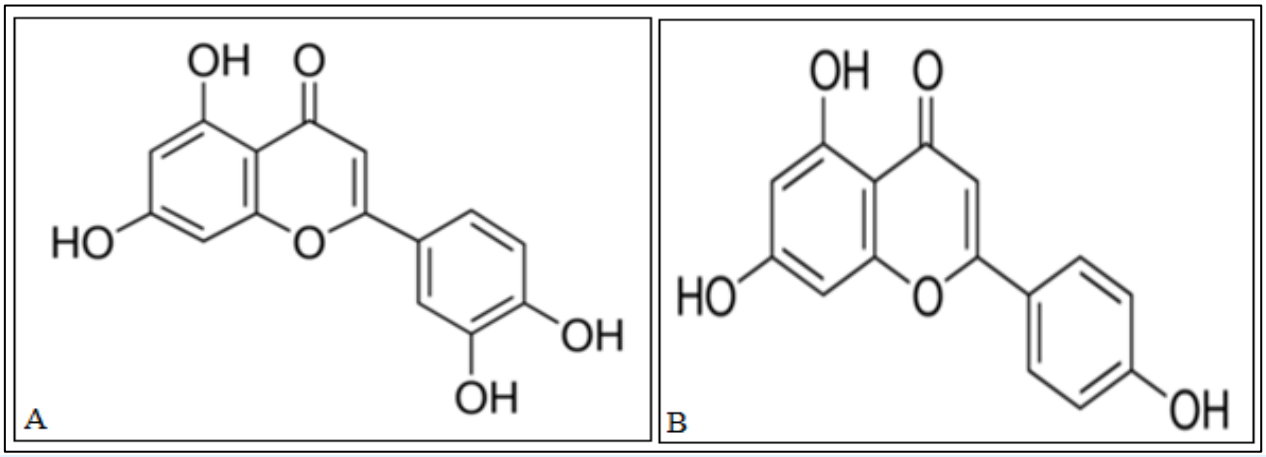

Fig. 1. Chemical structure of selected flavonoids: A: Luteolin, B:Apigenin

Table 2. Flavonoids test of Stachys sp. methanolic extract

Tests	Result
Shinoda test	+
Ferric chloride test	+
Lead acetate test	+

(+) Presence

Molecular docking method for DNA binding to flavonoids compounds in Stachys sp. Extract

Molecular docking experiments were performed with AutoDock 4.2 all parameters were set as defaults. The AutoDock docking procedures were composed of two steps: (1) using the Lamarkian Genetic Algorithm to sample the ligand conformation in the binding sites of selected DNA, based on the pre-calculated energy grids, where the binding site was defined as all atoms within 6Å of the cognate ligands, the grid spacing was set to 0.375Å, and the number of evaluations per docking run was 2,500,000; and (2) the AutoDock scoring function was subsequently used to determine the binding scores of the different conformations (Fong and Wong, 2019). The Protein Data Bank was searched for ligand-DNA complexes, and the structure was selected: 1N37 d(AGACGTCT)2 (Searle, Maynard and Williams, 2003). All ions, ligands, and water molecules were then removed and hydrogen was added before the docking simulations. We choose two common flavonoids in Stachys sp. methanolic extract according to literature apigenin and luteolin (Kharazian and Mohammadi, 2014 ; Vundać, 2019). Chemical structures of two flavonoids were downloaded from PubChem as a 3D conformer SDF file **Fig. 1**. **Table 1** illustrate Lipinski's physicochemical parameters rule for selected flavonoids.

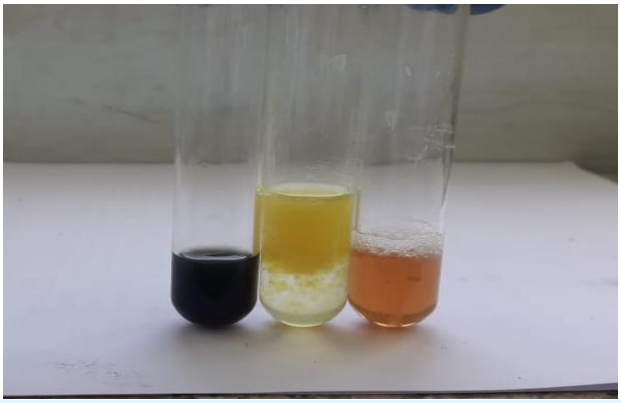

Fig. 2. Detection of flavonoids in *Stachys sp.* Methanolic extract

RESULTS

Flavonoids tests

The results of flavonoids tests of Stachys sp. methanolic extract leaves as reported in **Table 2** (**Fig. 2**) which showed the presence of flavonoids in the methanolic extract.

Determination of total flavonoids content

The total flavonoids for the Stachys sp. methanolic extract were 103.36 ±2.95 mg of quercetin/gm of methanolic extract.

Hydrogen peroxide scavenging assay

The scavenging ability of methanolic extracts of Stachys sp. on hydrogen peroxide is shown in (**Fig. 3**) and compared with ascorbic acid as standards. The result indicates the highest inhibition percentage was (38%) belongs to (2 mg/ml) of extract in comparison with (48.57%) for ascorbic acid, and the IC50 of Stachys sp. extract was 2.894 mg/ml.

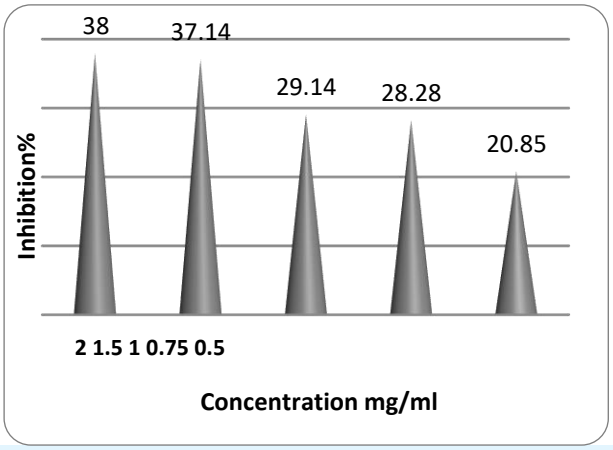

Fig. 3. Hydrogen peroxide radical scavenging activity of *Stachy sp.* methanolic extract

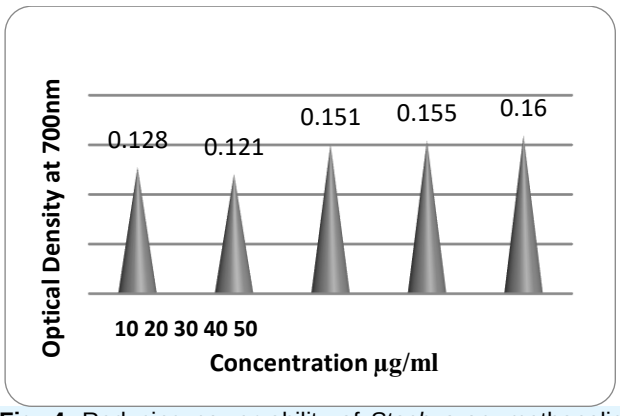

Fig. 4. Reducing power ability of *Stachys sp.* methanolic extract

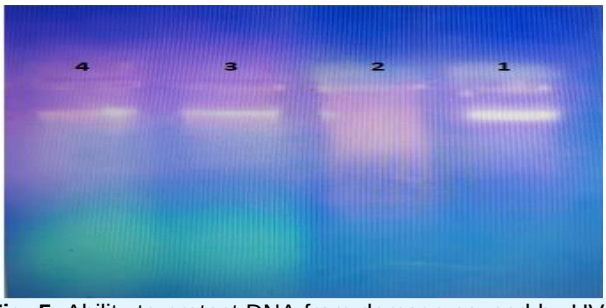

Fig. 5. Ability to protect DNA from damage caused by UV-light and (3% v/v) H2O2 : Lane 1: Control (untreated DNA), Lane2: DNA UV-irradiated and treated with (3% v/v) H2O2, Lanes 3 and 4 shows two concentration 2 and 1 mg/ml of Stachys sp. methanolic extract with DNA and exposition to UV-light and 3% v/v H2O2

Reducing power assay

The reducing power of the methanolic extracts of Stachys sp. was determined and the results are shown in (**Fig. 4**). The methanolic extract displayed high reducing power activity.

DNA damage protection assay

Results showed that the Stachys sp. methanolic extract at two concentrations (2 and 1 mg/ml) was exhibited completed protection of human genomic DNA (**Fig. 5**).

Table 3. Docking results for Apigenin and Luteolin with 1N37 DNA

Selected flavonoids	Binding free energy	Intermolecular energy	Electrostatic energy	Cluster RMSD
Apigenin	-6.86	-8.06	0.1	0.00
Luteolin	-7.11	-8.61	0.0	0.00

Table 4. Type of bonds formed due to interaction between Apigenin and Luteolin with 1N37

Ligands	Receptor (1N37)	Chain	Interaction	Distance	E (Kcal/mol)
Apigenin					
O 18	O3` DG 5	A	H-donor	3.01	-1.4
O 28	O4` DG 13	B	H-donor	2.87	-1.0
6-ring	6-ring DG 5	A	pi-pi	3.82	-0.0
6-ring	6-ring DG 13	B	pi-pi	3.36	-0.0
6-ring	5-ring DG 13	B	pi-pi	3.92	-0.0
Luteolin					
O 18	O3` DG 5	A	H-donor	2.89	-0.9
O 20	O3` DG 5	A	H-donor	3.17	-0.8
O 22	O4` DG 13	B	H-donor	3.13	-1.2
6-ring	6-ring DG 13	A	pi-pi	3.71	-0.0
6-ring	6-ring DG 5	B	pi-pi	3.80	-0.0
6-ring	6-ring DG 13	B	pi-pi	3.37	-0.0
6-ring	5-ring DG 13		pi-pi	3.77	-0.0

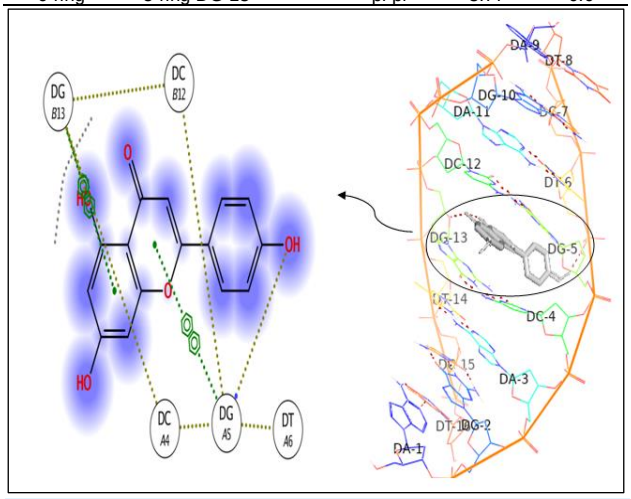

Fig. 6. Molecular docked complex of apigenin with 1N37 3D pose (right), interaction of apigenin with 1N37 2D image (left)

Molecular docking studies

Among ten conformations in each docking for apigenin and luteolin, one conformation was chosen. Ten conformations were analyzed and detect the best conformation depending on binding free energy. In **Table 3** it is reported a summary of the results for apigenin and luteolin dockings with DNA 1N37. **Table 4** shows the bonds forms between apigenin and luteolin with DNA (1N37) double helix after docking. (**Fig. 6** and **7**) illustrate the best-docked conformation for docking apigenin and luteolin onto 1N37 double helix.

DISCUSSION

From ancient time till now the medicinal plants act a treasure for many compounds such as flavonoids,

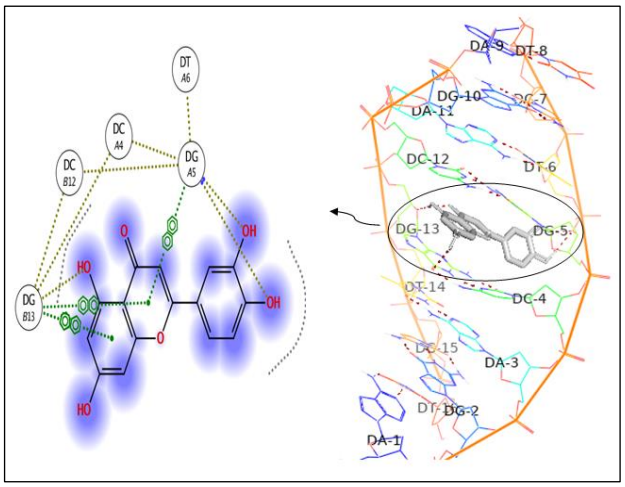

Fig. 7. Molecular docked complex of luteolin with 1N37 3D pose (right), interaction of luteolin with 1N37 2D image (left)

alkaloids, phenolic acid, and carbohydrates which have many activities to use it in medicine or industry. Flavonoids are an important class of natural products; particularly, they belong to a class of plant secondary metabolites having a polyphenolic structure, in the Labiatae (Lamiaceae) family, they occur in every part of the plants as in other families but are usually found in the aerial parts (Panche, Diwan and Chandra, 2016 ; Ulubelen, Topcu and Kolak, 2005). Stachys sp. is a genus of about 300 spp. In the mint family, Lamiaceae, it is the third-largest genus (113 spp.), in Iraq, there is (20 spp.) of this genus, the aerial parts of plants belonging to this genus have been used in folk medicine and phytotherapy for many years, many investigations of this taxa have revealed the presence of flavonoids (Hamodie, 2019 ; Bilušić Vundać et al., 2005), and this approved by flavonoids tests (**Fig. 2** & **Table 2**), and also methanolic extract contains a significant amount of flavonoids 103.36 mg. Flavonoids occur as glycosides and contain several phenolics hydroxyl groups, many flavonoids are found to be strong antioxidants effectively scavenging the reactive oxygen species because of their phenolics hydroxyl groups (Khan et al., 2012), (**Fig. 3**) shows scavenging activity of Stachy sp. methanolic extract which can be consoled to flavonoids content, the comprehensive model of action of flavonoids includes: quenching free radical elements, chelating metal, suppressing the enzymes associated with the free radical generation, and stimulation of internal antioxidant enzymes, The best-described antioxidant property of flavonoids derives from its ability to directly scavenge the reactive oxygen species, flavonoids can chelate free radicals immediately by donating a hydrogen atom or by single-electron transfer, another possible mechanism of action of flavonoids is through the chelation of transition metal elements, flavonoids have chelating property, which enabled them to chelate, or binds to metal ions in the human body to prevent them from being accessible for oxidation, flavonoids can also act as an intracellular antioxidant through inhibition of free radical generating enzymes (Banjarnahor and Artanti, 2014). (**Fig. 4**) illustrate the reducing power ability of Stachys sp. methanolic extract, The reduction capacity of a compound may serve as a significant indicator of its antioxidant activity, the antioxidant compounds are responsible for the reduction of ferric (Fe3+) form to ferrous (Fe2+) form, this reduction capacity can be determined by measuring the colored complex at 700 nm so the ability of Stachys sp. MtOH extract to reduce Fe3+ can be an interpretation by the presence of flavonoids that breaking the free radical chain through donating a hydrogen atom (Plaza et al., 2014 ; Labiad et al., 2017). From (**Fig. 5**) we can see the ability of Stachys sp. MtOH to protect human DNA by two concentration (2&1 mg/ml), the extract effectively ease the oxidative stress and protected the DNA from hydroxyl radicals and UV-radiation, polyphenol compounds such as flavonoids can reduce oxidative DNA damage through inhibition of ROS, flavonoids can form complexes with DNA and protect it against oxidative damage, the antioxidation potential of antioxidants are related to their binding modes to the DNA duplex (Kumar et al., 2013 ; Tiwari and Mishra, 2017). To explanation the binding mode of flavonoids to DNA we use a molecular docking study, which is the most suitable way for a theoretical understanding of the molecular mechanism and the elucidation of binding mode/modes of a compound with DNA through non-covalent interactions, this theoretical approach is considered most appropriate for structure-based- drug design, molecular docking studies along with experimental studies could help to explore a compound as a potential drug candidate (Arshad et al., 2018). We choose two flavonoids in Stachys as an example (apigenin, luteolin), and the auto-dock conformation with the best binding free energy (**Table 3**) illustrates that apigenin and luteolin form hydrogen bonds with DNA (specifically with guanosine 5 &13) (**Table 4**) (**Fig. 6** and **7**) when compared with the X-ray crystallographic conformation, the hydrogen bonding mode with DNA was found to be the same and that maybe explain the ability of flavonoids to protect DNA (Chen, Adelstein and Kassis, 2004). Finally, flavonoids are found to be rich in fruits, and vegetables, these are important components of both human and animal diets and are safe to be consumed, they exhibit a wide variety of biological activities like antiviral, antibacterial, anti-inflammatory, anti-carcinogenic, antiplatelet antioxidant, and DNA protection activities (Soumya et al., 2019).

CONCLUSION

This study is detailed research on the antioxidant activity, reduction capacity, and DNA protection ability for Stachys sp. methanolic extract which contains

flavonoids, The research also introduces a molecular docking study to explain the nature of the binding bond between DNA and two examples of flavonoids in Stachys sp. methanolic extract apigenin and luteolin

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