Experimental and Theoretical Investigation on the Scavenging of 2, 2-Diphenyl-1-Picrylhydrazyl Radical by Thymoquinone

By

KARTHIKA.S

(20PPH010)

A Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore-641043

In partial fulfilment of the requirement of the degree of

MASTER OF SCIENCE IN PHYSICS

MAY-2022

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CERTIFIED AS A BONAFIDE RESEARCH WORK

27/05/22

Signature of Head of the department

27/5/22

Signature of the supervisor

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KARTHIKA.S

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INTRODUCTION

CHAPTER 1

INRODUCTION

"Health" is a state of complete physical, mental, social and spiritual well- being and not just the non existence of illness of infirmity" is the primary criteria for leading the happy life. Diseases on the other hand is " A pathological or patho physiological state of a single part, whole organ or complete system of an organism consequentially developing from a variety of reasons such like contagion, hereditary defect or ecological stress and branded by a exact set of signs or symptoms". Human being on this planet suffers from various catagories of diseases naming infectious diseases, autoimmune diseases, mutational diseases; drug induces diseases which will be cured by the application of therapeutic agents. During early age, man learned the method of using plants and its different parts are medicine, later with the advent of science and technology individual molecules were separated and tested for the therapeutic potency which propelled mankind towards better future. Although various allopathic medicines like insulin the future runners in the treatment of diabetes and pyrexia, but on the other hand they have serious side effects and gives rise to complications. As the intensity of this disorders increase, there is need to look for drug with satisfying results and less side effects.

1.1 Natural plants

Plants that possess vast medicinal properties that show beneficial pharmacological effects on the human body are generally designed as medicinal plants [1]. Medicinal plants are used in pharmaceutical industries, medicines nutraceuticals and food supplements. Herbal medicine from different plants and their parts are used tradicinally in medicine to treat various diseases and disorders [2].Bio active constituents of plants known as phytochemical components such as tannins, carbohydrates, alkaloids, terpenoids, phenolic compounds, steroidsand flavonoids are responsible for various pharmacological activities of the plants. These Phytochemical compounds are synthesized by primary or secondary metabolism of living organisms [3]. Plants are important to the global economy involved in formulation of drugs with cheaper resources without side effect [4]. The present research is aimed to study the following medicinal plants with their phytochemical screening and antioxidant potential.

1.1.1 Free radicals

Free radicals are chemical species possessing an unpaired electron that can be considered as fragments of molecules and which are generally very reactive. As such, free radicals can be formed in three ways: (i) by the homolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the paired electrons; (ii) by the loss of a single electron from a normal molecule; (iii) by die addition of a single electron to a normal molecule.] The most important free radical which consists of oxygen are hydroxyl radical, Superoxide anion radical, Hydrogen peroxide, Nitric oxide radical, Peroxyl radical.[5].

1.1.2 Superoxide radical

Superoxide is an anion radical produced by one electron reduction of molecular oxygen. The reactive oxygen anion superoxide is the product of one electron reduction of di oxygen O_2. It is diradical containing two unpaired electrons, addition of an electron fills one of the two molecular orbitals, learning unpaired electron with charge of -1.

1.1.3 Hydrogen peroxide radical

The hydroperoxyl radical, which is not so effective an oxidant as the hydroxyl radical, does not attack aliphatic alcohols; accordingly aldose is obtained from the higher aldonic acid. In the presence of an excess of hydrogen peroxide, however the accumulation of ferrous ions in solution catalyzes the production of hydroxyl radicals.

1.1.4 Hydroxyl radical

The hydroxyl radical ($HO^{\cdot}$), the three electron reduction product of molecular oxygen, is the most reactive species of oxygen. It has a half life in biological system of about 1 ns and reacts with organic molecules. This high reactivity of $HO^{\cdot}$ Limited to no more 50 molecular diameters from the site of formation.

1.1.5 Nitric oxide

The generation of Nitric oxide is frequently coupled to activation of the NMDA class of glutamate receptor. Once produced, no diffuses rapidly across membranes an to act on neighboring cells, its principle receptors being specialized gyranylyl cyclase-coupled proteins. The receptors are tuned to detect subnanomolar concentrations of Nitric oxide and rapidly transduce them into micromolar concentrations of cyclic GMP. This pathway participates in numerous physiological functions, including synaptic plasticity. Though other mechanism, Nitric oxide contribute to neuropathology.

1.1.6 Peroxyl radical

Peroxyl radical are formed due to attack by molecular oxygen, which can abstract a hydrogen atom from a double bond in fatty acid site chains, producing a lipid hyroperoxide or lipid endoperoxide [6].Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Targets of free radicals include all kinds of molecules in the body. Among them, lipids, nucleic acids, and proteins are the major targets.In order to prevent the propagation of radicals, need to powerful protective scavengers like synthetic and natural antioxidants are necessary. Hence the use of natural antioxidants from plant sources which acts as defense system in the human body are free from any side effects has gained much attention nowadays.[5].

1.2 *Nigella sativa L.*

The common name and genus name *Nigella* is derived from the Latin *niger*, meaning "black." The term *nigellus* is a derivative that means "blackish" or "dark." The species name *sativa*, meaning "cultivated," is not surprising given that *Nigella sativa L.* had been cultivated for thousands of years before Swedish botanist Carl Linnaeus named it in 1753. *Nigella sativa L.* is also known as black cumin, black caraway, and black seed, *Nigella sativa L.* is an annual flowering plant. It grows to 20–30 cm (7.9–11.8 inch) tall and has linear lanceolate leaves. The delicate flowers have 5-10 petals and the colors are usually yellow, white, pink, pale blue or pale purple.[7]. *Nigella sativa L.*, often known as fennal flower plant, is an indigenous herbaceous plant that belongs to the Buttercup or Ranunculaceae family [8]. It grows in nations surrounding the Mediterranean Sea, such as Pakistan and India, and is native to Arab countries and other areas of the Mediterranean region [9].The *Nigella sativa L.* seed (*NSE*) are of particular interest because they contain phytochemicals with significant antioxidant properties and health benefits. The seeds are rich in oil, which contribute to human health and also nutrition due to major (essential fatty acids) and minor compounds (phenolic compounds, tocopherols, and sterols). [10]

NSE is one of the most well-known plants in the world. The chemical content of the seeds is extremely rich and diverse [11]. *NSE* seeds have traditionally been utilized in both food and "medicine" [12]. The fixed oils of *NSE* exhibit several pharmacological effects, including antioxidant, anti-inflammatory, immunomodulatory, anti-cancer activities and anti-diabetic drug that also have a major impact on the cardiovascular and gastrointestinal systems. We focused our investigation on the phenolic component of *NSE* in quest of physiologically active molecules.

1.2.1 Morphology of *Nigella satia L.*

The *Nigella sativa L.* plant is a green colour with finely divided linear leaves. The flower is pale blue and white in colour with 5-10 petals. The fruits are found in the form of inflated capsules. The capsules are further divided into 3-7 united follicles. Each follicle contains numerous *Nigella sativa L.*seeds which are oval in shape and black measuring about 1 mm in diameter. [13]. Fig 1.1 shows that the species grow to 20-30 cm tall, wherein the leaf segments are narrowly linear to threadlike. The flowers grow terminally on its branches. *Nigella sativa L.* reproduces with itself and forms a fruit capsule which consist of many white trigonal seeds, once the fruit capsules has matured, it opens up and the seeds contained within are exposed to the air becoming black in colour that is reason it also called as black seeds, seeds are triangular in shape, black in colour and possess a severe pungent smell, contains considerable amount of oil [14]. This plant is known by numerous names, for example black cumin (English), Black caraway seeds (USA), Shonaiz (Pakistan) and Kalajira (Bangali)

Fig 1.1 Different parts of *Nigella sativa.L*

1.2.2 Chemical constituents and active principles in *nigella sativa L.* seeds

The seeds are very rich and diverse in chemical composition, which contain amino acids, carbohydrates, fixed and volatile oil [15]. *Nigella sativa.L* also contains a good amount of various vitamins and minerals like Cu, P, Zn, and Fe. Many active compounds have been identified in *Nigella sativa.L* is attributed to Quinone constituents in the seed. The most important active compounds of *Nigella sativa.L* are thymoquinone, thymohydroquinone,

dithymoquinone (nigellone), carvacrol and thymol. *Nigella sativa L.* is also containing other compounds such as carvone, limonene, citronellol in trace amounts and two varieties of alkaloids (e.g. nigellidine and nigellicimine-N-oxide) and pyrazole alkaloids (e.g.nigellidine and nigellicine). The pharmacological properties of *Nigella sativa L.* are mainly attributable to its quinine constituents, TQ being the most abundant [16]. The *Nigella sativa L.* seeds contain fatty acids, constituting linoleic acid, eicosadienoic acid and saturated fatty acids. Most of the studies on the biological effects of *Nigella sativa L.* have dealt with its crude extracts in different solvents; however some studies used its active principles. Among the components isolated from the crude extract of *Nigella sativa L.*, thymoquinone (TQ) has been shown to be the principle active ingredient and thus is the most studied of all [17].

1.3 Quinones

Quinones belong to the group of quinoid compounds that are widely distributed in nature. Quinones have had two carbonyl group in the para-position of a ring, represent an important class of versatile organic compounds endowed with rich and fascinating chemistry. In the biological system like respiration and photosynthesis incorporate isoprenoid quinones as the integral part of the electron and energy transport chain in mitochondria and chloroplast [18]. Quinone can be formed from phenolic compounds either by acetate, affording a catechol or quinol system [19].

Quinones are secondary metabolites isolated from plants with structure of aromatic (hexacyclic saturated) di-one or di-ketone system, which derived from the oxidation of hydroquinones. Naturally occurring quinones are widely distributed and include; Fig 2.1 shows that the types of quinones.

(i) Benzoquinones
(ii) Naphthoquinones
(iii) Anthraquinones
(iv) Polyquinones.

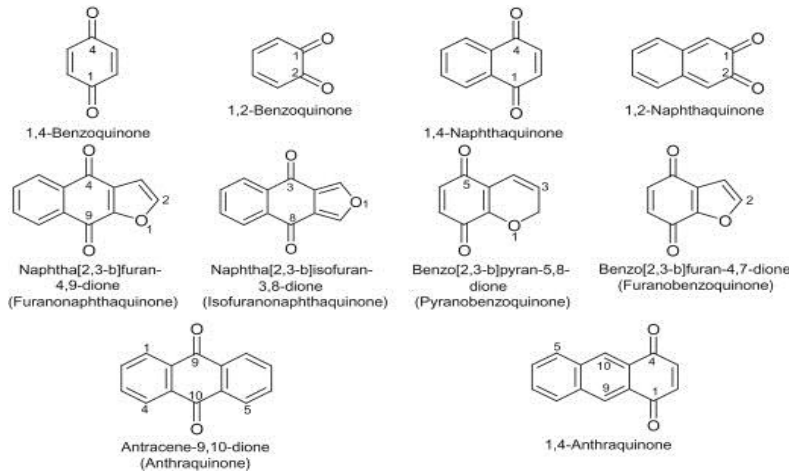

Fig.1.2. Schematic diagram of types of Quinones

1.3.1 Benzoquinones

Benzoquinones are groups of compounds containing two carbonyl groups on a saturated hexacyclic aromatic ring system (benzene ring), at ortho or para positions (monocyclic).

1.3.2 Naphthoquinones

Naphthoquinones contain the naphthalene nucleus with two carbonyl groups on one nucleus, usually at the ortho or para position (bicyclic) and occur at fungi and in plants.

1.3.3 Anthraquinones

The compounds containing the anthracene nucleus with two carbonyl groups, usually on ring B at para positions (tricyclic).

1.3.4 Polyquinones

polyquinones are dimers which had inter- or intramolecular oxidative coupling with formation of carbon–carbon or carbon–oxygen bonds [20].

1.4 Quinones in *Nigella sativa L.*

Quinones are found in a wide variety of plant families such as Ranunculaceas, Aphodelaceas, Fabaceae, Ebenaceae and Rhamnaceae. Shows the quinones types of compound identified in *Nigella sativa L.* (Ranunculaceas family). Many active compounds have been isolated, identified and reported in different varieties of *Nigella sativa L.*seeds. Fig 1.3 shows the most important active compounds are thymoquinone, thymohydroquinone, dithymoquinone , thymol, and carvacrol *Nigella sativa L.*seeds also contain some other compounds in trace amounts. Seeds contain two different alkaloids i.e isoquinoline alkaloids

e. g. nigellicimine and nigellicimine-N-oxide, and pyrazol alkaloids or indazole ring bearing alkaloids which include nigellidine and nigellicine [21].

1.4.1 Thymohydroquinone

Thymohydroquinone (1,4-benzenediol, 2-methyl-5-(1-methylethyl) is a component of Black cumin seed oil and thyme. It is also a reduction product of thymoquinone. Which is a phytochemical compound found in the plant *Nigella sativa L*. Thymoquinone exhibits antioxidant and analgesic properties.

1.4.2 Dithymoquinone

Dithymoquinone (4b,8b-Dimethyl-3,7-di(propan-2-yl)-4a,8a-dihydrobiphenylene-1,4,5,8-tetrone) is a bioactive isolate of *Nigella sativa* L. Chemically, it is a dimer of thymoquinone.

1.4.3 Thymol

Thymol (2-isopropyl-5-methyphenol 5-methyl-2-isopropylphenol) is a phenol and had cymene derivative from natural.

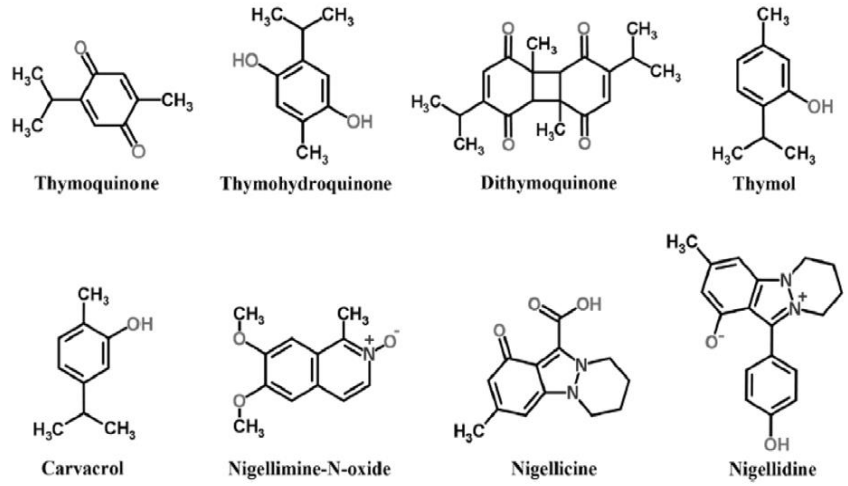

Fig1.3. Chemical constituents found in *Nigella sativa.L seed* extracts

1.4.4 Carvacrol

Carvacrol (5-isopropyl-2-methylphenol) is a phenol and natural monoterpene derived from cymene. Bacterial growth is used a food additive to inhibit.

1.4.5 Nigellimine-n-oxide

Nigellimine N-oxide (6,7-dimethoxyl-1-methyl-2λ^5-isoquinolin-2-one) belongs to the class of organic compounds known as isoquinolines which had aromatic polycyclic compounds containing an isoquinoline moiety, consists of a benzene ring fused to a pyridine ring and forming benzo pyridine.

1.4.6 Nigellicine

Nigellicine(9-hydroxy-7-methyl-5-pyridazino[1,2-a]indazol-11-ylium-10-carboxylate) is found in herbs and spices. Nigellicine is an alkaloid from the seeds of *Nigella sativa L.* [22].

1.4.7 Nigellidine

Nigellidine(6,7,8,9-Tetrohydro-1-hydroxyl-11-(4hydroxyphenyl)-3methylpyridazino[1,2-a]indazol-5-ium inner salt,9cl) which is found in *Nigella sativa L.* seeds.

1.4.8 Thymoquinone

Thymoquinone (TQ) (2-isopropyl-5-methyl-1,4-benzoquinone),shows in table 1.1 which has the chemical formula $C_{10}H_{12}O_2$ and a molecular weight of 164.2 g/mol, with major phytochemical bioactive ingredient in *Nigella sativa L.* oil and extracts [23].The chemical structure of TQ is shown in Fig. 1.4.

Table 1.1 Physicochemical properties of TQ

IUPAC Name	2-isopropyl-5-methylbenzo-1, 4-quinone
Chemical formula	C10H12O2
Molecular formula	164.20g/mol
Appearance	TQ is dark yellow crystalline powder
Melting Point	45-470C

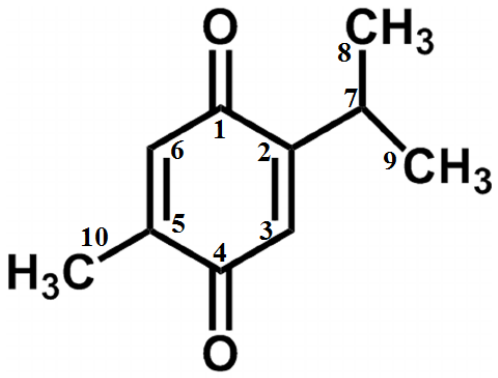

Fig.1.4. Structure of Thymoquinone

Herbal medicine has attracted great attention in the recent years and and is increasingly used as alternatives to chemical drugs. Several lines of evidence support the positive impact of medicinal plants in the prevention and cure of a wide range of diseases .TQ is the most abundant constituent of the volatile oil of *Nigella sativa L.* seeds and most properties of *Nigella sativa L.* are mainly attributed TQ. A number of pharmacological actions of TQ have been investigated including anti-oxidant, anti-inflammatory, immunomodulatory, and anti-histammic, anti-microbial and antitumor effects. It has also gastroprotective, hepatoprotective, nephroprotective and neutroprotective activities. In addition, a large body of data shows that TQ has very low adverse effects and no serious toxicity. More recently, a great deal of attention has been given to this dietary phytochemical with an increasing interest to investigate it in preclinical and clinical researches for assessing its health benefits. The analyze of numerous properties of the active ingredient of *Nigella* sativa L. seeds, TQ in the context of its therapeutic potentials for a wide range of illnesses. TQ is known to be the primary active constituent on *Nigella sativa L.* seeds responsible for its medicinal effects and also showing promise for treatment of cancer. TQ is the bioactive compound derived from *Nigella sativa* L. the seed is reportedly associated with diverse therapeutic benefits to bronchial asthma, dysentery, headache, gastrointestinal problems, eczema, hypertension and obesity [24]**.**

1.4.9 Free radical scavenging and antioxidant potential of thymoquinone

Thymoquinone**(**TQ) is a short-chain ubiquinone derivative that potentially acts as a pro-oxidant it has been hypothesized that inflammation and pro-oxidant milieu is actually something that is our bodies own creation, an adverse by product effect of the essential metabolism and inflammatory system that protects us against diseases.[25].Its broad spectrum antioxidant potential is associated with its potential to alter “redox state” and its scavenging

ability against free radicals, including reactive oxygen species (ROS; superoxide anion radical, hydroxyl radical, hydrogen peroxide, peroxynitrate) through modulation of hepatic and extra hepatic antioxidant enzymes which as superoxide dismutase, catalase and GPx .

1.5 2, 2-diphenyl-1-picrylhydrazyl (dpph$^{\bullet}$)

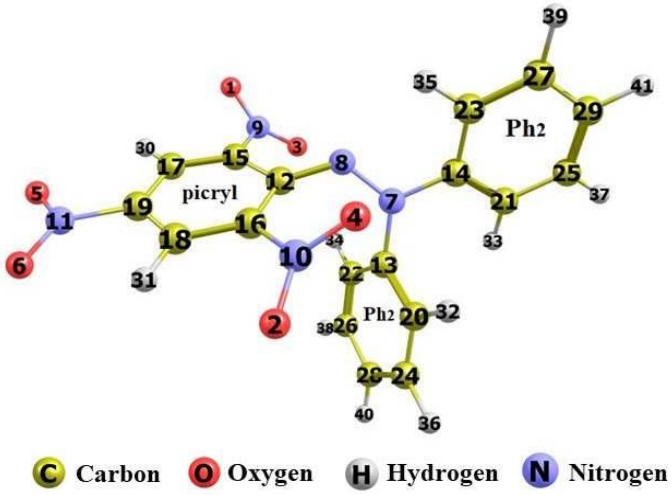

Fig1.5 The optimized structure of DPPH $^{\bullet}$

The stable aromatic free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) is one of the most commonly used standard compound to test the antioxidant activity by the in-vitro method DPPH$^{\bullet}$ is composed of two phenyl rings (Ph_2) interconnected by N=N with picrylmoiety (NO_2) shows in fig 1.5. The no polar DPPH radical has a C1 symmetry with one unpaired electron, mainly located on the central nitrogen atom used the hybrid density functional theory to study the solvent interaction of methanol with DPPH through hydrogen bonding. It suggests that the interaction the position of picrylortho-nitro groups had a more significant effect on the orientation of phenyl ring with respect to NO2 ring, and confirms the presence of unpaired electrons at the nitrogen centered group(N=N).DPPH reduced to form the stable neutral DPPH-H molecule by accepting a hydrogen atom or electrons from the interacting antioxidant. The reaction mechanisms between some antioxidants and free radicals have been studied to evidence the HAT and SET mechanism through computational investigation reported that the reaction between quercetin and free radicals, OH^- and $O2^-$radicals from quercetin, which follows the mechanisms HAT and SET, and the reactivity of quercetin was primarily due to the reaction between fisetin and DPPH are highly directed

by the hydrogen atom transfer from the most active site 4'-hydroxyl group(OH) through HAT mechanism. Moreover, the attack by on antioxidant the DPPH radical was an exothermic reaction. Reported that the flavanol shave an advantage in reducing the cholesterol content in micelles through charge transfer from the lone fair of oxygen atom(O) of aromatic rings of the thymoquinone to the hydrogen atom(OH) of cholesterol, followed by hydrogen bond interaction (O-H…O). The strength of the hydrogen bond determines the stability of complexes through intermolecular charge transfer. [26]

1.6 Objective of the present work

- To extract the *Nigella sativa L.* (*NSE)* seed using Methanol and water
- To identify Thymoquinone (TQ) in *NSE* by High Performance Liquid Chromatography (HPLC**)**
- To evaluate the antibacterial activity of *NSE* and TQ using gram positive and negative bacteria.
- To investigate the antioxidant activity of thymoquinone was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$**)** assay
- The optimized structures of TQ and DPPH$^{\bullet}$ forms the complex TQ-DPPH$^{\bullet}$ using DFT with the B3LYP/6-311+G (d, p) method.
- To analyze the interaction energy of TQ-DPPH
- To analyze the quantum theory of atoms in molecules (QTAIM), natural bond orbital (NBO) and topological analysis using the wave functions computed at the B3LYP level of theory
- To evaluate the frontier molecular orbital energy values and global descriptors is calculated by B3LYP method with 6-311++G (d, p) basis set.
- The binding pocket of BSA is identified with the interaction of TQ using molecular docking studies.

REVIEW OF LITERATURE

CHAPTER – II

REVIEW OF LITERATURE

2.1 Introduction

This chapter deals with review of literature on the extraction from NSE using different methods. And the optimized geometrical parameters of TQ with free radicals were investigated the interactions between the complexes.

2.2 Overview of literature

Jewel Hossen et.al.,(2021) reported that thymoquinone was a natural compound present in black cumin which possesses potent antioxidant activity without having any phenolic hydroxyl group which is responsible for antioxidant activity, computational calculation based on density functional theory (DFT) was executed to assess systematically the antioxidant behavior of this compound by considering geometrical characteristics, highest occupied molecular orbital - lowest unoccupied molecular orbital (HOMO-LUMO), and molecular electrostatic potential (MEP) surface. Thermo chemical parameters correlated to the leading antioxidant mechanisms such as hydrogen atom transfer (HAT), single electron transfer-proton transfer (SETPT), and sequential proton loss electron transfer (SPLET). In addition, the changes of thermo chemical parameters such as free energy change (ΔG) and enthalpy change (ΔH) were computed for hydrogen abstraction (HA) from TQ to hydroxyl radical in gas and water phases to investigate its free radical scavenging potency. The low and comparable values of bond dissociation enthalpy (BDE), proton dissociation enthalpy (PDE), ionization potential (IP), proton affinity (PA), and electron transfer enthalpy (ETE) revealed the antioxidant activity. The ΔG and ΔH also indicated apposite thermodynamic evidence in favor of antiradical capability of TQ. The attack of the free radical occurred preferentially at 3CH position of the molecule.

Thuy Phan Thi et.al., (2020) have investigated that Trans-resveratrol establishes the planarity in its structure which makes it an interesting compound in both experimental and theoretical examinations, using the density functional method (DFT), attempts to compare the anti oxidative capacities between hydroxyl (OH) and aromatic methane (CH) groups of this molecule. Becke's exchange-correlation B3LYP functional together with 6-311++G(d, p) basis set was used to reveal the effects of structural geometry and electronic feature on the

antio xidative results of OH and CH groups. The anti oxidative action of trans-resveratrol has followed the HAT mechanism in gas, but the SPLET pathway in liquids. OH bond breaking is easier than CH bond disruption. 4-OH bond breaking induces the lowest BDE values of 74.4–77.9 kcal/mol in gas, acetone, methanol, and water, as well as the lowest PA values of 37.2–46.2 kcal/mol in acetone, methanol, and water. From the kinetic view, 4-OH is also an active center to capture laboratory radical DPPH, ROS radicals $HOO^{\bullet}$ and $CH_3O^{\bullet}$, and RNS radical $^{\bullet}NO_2$.

Maciel, Eduardo N.; Soares, Iuri N et.al.,(2019) have studied the antioxidant potential of flavonols through the explicit modeling of chemical reactions. A theoretical investigation on the reaction between fisetin and 2,2-diphenyl-1-picrylhydrazyl (DPPH) is presented. All the computations were performed using the density functional theory with the B3LYP functional along with the 6-31G (d,p) basis set. Structural, energetic quantities (ΔG and ΔG++), and reaction rates were probed in order to provide information on the antioxidant activity and to explore the contributions of each hydroxyl group to the referred property, the order of contribution of each OH group to the antioxidant potential was found to be 4′-ArOH (the most contributor, presenting ΔG = -5.17 kcal/mol) → 3′-ArOH (ΔG = -3.35 kcal/mol) → 3-ArOH (ΔG = -1.64 kcal/mol) → 7-ArOH (ΔG = 7.72 kcal/mol). These observations are in consistent agreement with the outcomes of other computational investigations performed using bond dissociation enthalpies (BDEs) as descriptors for the antioxidant activity. Therefore, the methodology employed in this work can be used as an alternative for probing antioxidant potential of compounds derived from fisetin.

Zheng, Yan-Zhen; et., al(2019) reported that the flavonoids are vital constituents of propolis that are responsible for its medicinal activity. Flavonoid extraction commonly employs ethanol and water as solvents. In the extraction reaction, hydrogen-bonding interactions play a crucial role, hydrogen-bonding interactions between myricetin—an abundant flavonoid in propolis—and ethanol or water were studied theoretically using density functional theory (DFT) methods. The molecular geometry and charge properties of the myricetin monomer were analyzed first. After careful optimization, nine stable myricetin−CH_3CH_2OH/H_2O complex geometries were obtained. Hydrogen bonds were confirmed to exist in these optimized structures. The most stable structures were found to be those with hydrogen bonds involving the hydrogen atoms of hydroxyl groups and the oxygen atom of the keto group of myricetin. The hydrogen bonds in the optimized geometries were shown to be closed-shell-type interactions. H5′ in ring B of myricetin presented the strongest interaction. Those between the hydrogen atoms of the hydroxyl groups in myricetin and the oxygen atoms in

CH_3CH_2OH and H_2O were of moderate strength and had some covalent character, while the others were weak and were dominantly electrostatic in character.

Nurulain Syamimi Mohamad et.al., (2018) have explored the efficiency of two different extraction method namely was aqueous and methanol extraction was successfully developed through TQ screening analysis and characterization through HPLC analysis. The *Nigella sativa* seed contain a bioactive compound is TQ which was prominent for its pharmacological properties such as antioxidant, anti-microbial, antibacterial and anti-inflammatory and its aim to extract and characterize TQ from *Nigella sativa* based products. TQ was isolated from raw *Nigella sativa* seeds using methanolic and aqueous extraction methods. Extracts were subjected to qualitative and quantitative analysis using High-Performance Liquid Chromatography.

RCN Thilakarathna et.al., (2018)have checked the phenolic content and antioxidant activities of methanolic extracts of *Nigella sativa* seeds relative to Ethiopian and Indian black cumin were examined. Ferric thiocyanate test was carried out to find the reducing power of both types of *Nigella sativa* seeds. This test was utilized in explaining antioxidant activity of methanolic extract of seeds. The phenolic composition of the methanolic extract was estimated by Folin-Ciocalteu spectrophotometric method. Significantly higher amount of total phenolic content was exhibited by Ethiopian Black cumin seeds compared to Indian type with a mean value of gallic acid. Similarly DPPH radical scavenging activity to gallic acid equivalents per ml respective Ethiopian and Indian *Nigella sativa*Seeds was observed. In addition ABTS radical scavenging assay showed and were recoreded in Ehiopian and Indian *Nigellasativa*seeds respectively.

Zuridah H., Fairuz A.r.M., et.al., (2008) found that *Nigella Sativa* seeds (NSE) have been used for medicinal purposes for centuries both as herbs and its oil. In Islam it is regarded as one of the greatest forms of healing medicine included in the medicine of prophet Mohammed. A number of these investigations emphasized the antimicrobial effect of them by using different extracts. They have tried to use the normal human mechanism in digestion by using the ground seeds. A modified paper disc diffusion method was used to test the antibacterial effect of NS seeds. Clear inhibition of the growth of Staphylococcus aureus was observed by concentration of 300mg/ml with distilled water (D.W.) as control , this inhibition was confirmed by using the positive control Azithromycin. The inhibition obtained was higher with Nigella sativa ground seeds from Hadramout (HNSGS) than with *Nigella sativa l.* ground seeds from Ethiopia (ENSGS). No inhibition was found in the growth of E.Coli and

Enterobacter. This was emphasized by using the positive control Ciprofloxacin. The positive inhibition may be attributed to the two important active ingredients of NS, Thymoquinone and melanin .

Nida Habib et.al., (2021) investigated that the medicinal importance of *Nigella sativa l.* seeds for treating various ailments is portrayed by its traditional uses. Owing to its immense pharmacological importance, the thymoquinone phytoconstituent of *N. sativa* can prove beneficial for the South Asian countries including Pakistan, where this seed is commonly produced and healthcare facilities are limited. In this study, the antibacterial activity of various extracts of *N. sativa* seeds, extracted thymoquinone, and oil samples have been investigated against *Bacillus subtilis* and *Bacillus licheniformis* using well and disc diffusion assay. The inhibition zones ranged between 7 and 44 mm against both the bacterial strains by well diffusion assay, while disc diffusion assay provided inhibition zones in the range of 7–23 mm. The crude extracts from 10 g of these seeds were subjected to preliminary phytochemical investigation.. Thymoquinone, a therapeutically important bioactive in *N. sativa* seed, was extracted employing both solvents. TLC assay and UV spectroscopy were used for its qualitative assessment, while HPLC-UV quantification showed that 250 mg/mL of methanol extract had 368.3 μg/mL thymoquinone, while its successive extraction yielded 32.94 μg/mL thymoquinone.

Mwadham M. Kabanda, et.al., (2021) reported that the potent antioxidant activity of flavonoids relevant to their ability to scavenge reactive oxygen species is the most important function of flavonoids. Density functional theory calculations were explored to investigate the antioxidant activity of flavonoid compounds such as apigenin and scutellarein. The biological characteristics are dependent on electronic parameters, describing the charge distribution on the rings of the flavonoid molecules. The computation of structural and various molecular descriptors such as polarizability, dipole moment, energy gap, homolytic O–H bond dissociation enthalpies (BDEs), ionization potential (IP), electron affinity, hardness, softness, electronegativity, electrophilic index and density plot of molecular orbital for neutral as well as radical species were carried out and studied. The B3LYP/6-311G(d,p) basis set was adopted for all the computations. This computation reveals that scutellarein exhibits higher degree of antioxidant activity than apigenin. Their dipole moment and polarizability analysis show that both the compounds are polar in nature and have the capacity to polarize other atoms.

S. Mohamad Reza NazifI et.al.,(2019) have studied the antioxidant activity of three *Aloe vera* components (aloesone, aloe-emodin, and isoeleutheol) was performed based on density functional theory calculations using the B3LYP hybrid functional and the 6–311++ G** basis set. Calculation of highest occupied molecular orbital (HOMO), lowest occupied molecular orbital (LUMO), and E_{gap} revealed that aloe-emodin has the lowest E_{gap} value, indicating good antioxidant activity. Also in terms of electron affinity, softness, electrophilicity, and chemical potential, aloe-emodin is a potent structure with potential high radical scavenging activity. Calculation of the ionization potential revealed that isoeleutherol likely also possesses a high degree of antiradical scavenging. To study the conjugating system of the radicals, density plots of HOMO, natural bond orbital analyses, and spin density plots were used. According to calculations, the isoeleutherol radical is more delocalized and the most stable radical. Calculated proton affinity values revealed that the most probable antioxidant mechanism is sequential proton loss-electron transfer.

Emre Çakmak & Dilara Özbakır Işın et.al.,(2020) reported that the Chromone (4H-chromen-4-one, 4H-1-benzopyran-4-one) and related compounds are important pharmacophores and privileged structures in medicinal chemistry because of their important biological activities such as anti-tumor, anti-HIV, and antioxidant. In the study, the density functional theory (DFT) calculations were performed for radical scavenging activity evaluation of a series of 3-styrylchromone derivatives. The reaction enthalpies related to the steps in the radical scavenging action mechanisms and several physicochemical descriptors such as global hardness, softness, and electronegativity were computed in gas phase and in water. The solvation effect of water on the antioxidant activity was taken into account by using the conductor-like polarizable continuum model. The calculations considering all physicochemical properties of molecules: thermodynamic, orbital, and structural. The results obtained were consistent with the experimental results.

Shabina Ishatiaq et.al., (2013) have investigated to find in vitro antibacterial activity of *Nigella sativa* extracts against seven clinical isolates identified by ribotyping. Crude extracts of *Nigella sativa* in eight organic solvents (Aqueous, Methanol, Ethanol, Chloroform, Butanol, Diethyl ether, n-Hexane and Acetone) were evaluated at 5 different concentrations 100 mg/ml , 50mg/ml , 25mg/ml , 10mg/ml and 5mg/ml by using disk diffusion method against human pathogenic bacterial strains including gram positive bacteria and gram negative bacteria. All the organic *N.Sativa* extracts with minimum concentrations of 5mg/ml showed effective growth inhibitation aginst the tested pathogenic bacterial strains. Out of the

eight extracts showed maximum antibacterial activity. The study will provide an insight to characterize bioactive compounds from these extracts, which can acts as strong bacterial growth inhibitor against wide range of infectious disease caused by pathogenic bacteria [36].

Prawez Alam et.al., (2013) have developed a sensitive and accurate high-performance thin layer chromatography method and to determine the quality of TQ in two different *N.sativa* extracts and marketed formulations. TQ was separated on aluminium backed silica gel with n-hexaneethyl acetate as mobile phase. A compact band was obtained for TQ calculated Rf value. This study concluded that to developed high-performance liquid chromatography densitometric method was sound cheap, selective, precise and accurate and can be used for routine analysis of *N.sativa* extracts and marketed formulations [37].

Muzaffar Iqbal et.al (2013) have estimated a simple, economic, robust, reproducible, selective, and precise high-performance liquid chromatography (HPLC) method developed and validated for the estimation of TQ in two different extract and marketed formulations. The mobile phase composed of 20-mM KH2PO4 buffer and acetonitrile at a ratio of 60:40 eluted at a flow rate of 1 mL/min. TQ was monitored using florescence detector set at 274 excitation and 340 emission wavelength, having column oven temperature of 40°C and sample cooler temperature of 8°C ± 0.2°C. The linear regression analysis data for the calibration curve shows good linear relationship with correlation coefficient of 0.998 in the concentration range of 0.07- 12 μg/mL. The limit of detection and limit of quantification were 0.023 and 0.07 μg/mL, respectively. The developed method was validated for accuracy, precision, reproducibility, and robustness as per ICH guidelines. The proposed method with high degree of precision and accuracy is employed for the estimation of TQ in methanolic and petroleum extract of Nigella sativa as well as in formulation. Statistical analysis proved that the method is precise, reproducible, selective, and accurate for the estimation of TQ for quality control purpose [38].

METHODOLOGY

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter covers both the experimental and theoretical methodologies used in this study. The *Nigella sativa L. seed (NSE)* was used to extract crude. *NSE* extract and Thymoquinone (TQ) is used for High performance liquid chromatography, antibacterial activity and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay analysis. The relation between structure and electronic properties of TQ is investigated using molecular descriptors. The Topological and Natural Bond Orbital's (NBO) analysis was carried out to evaluate stabilization energy of the molecule. Docking studies to examine the presence and interaction of the complexes.

3.2 Experimental methodology

3.2.1 Materials and methods

3.2.1.1 Materials

- *Nigella Sativa.L*
- Hexane
- Methanol
- Thymoquinone-Sigma Aldrich

3.2.1.2 Extraction of *NSE*

In the present study, *Nigella sativa L.* is collected from Palamudhir shop, Coimbatore. The black cumin seeds were cleaned with fresh water to remove dust, and then dried for four days at room temperature. After that the seed crushed into powder using grinder and store in refrigerator for further use [41].

3.2.1.3 Ultrasound assisted extraction

The 25 gram of *NSE* powder defatted by ultrasonic aided extraction is combined with 250 ml of Methanol in a beaker immersed in an ice bath and sonicated for 45 mints at frequency with 50w power. The solvent was extracted by a rotary evaporator at 50°C after the extraction was filtered through Whatman no.1 filter paper. For 15 days, the extraction was

stored in a freeze drier at for future use, the powder *NSE* extract is kept at 40° shows in fig 3.1 [42].

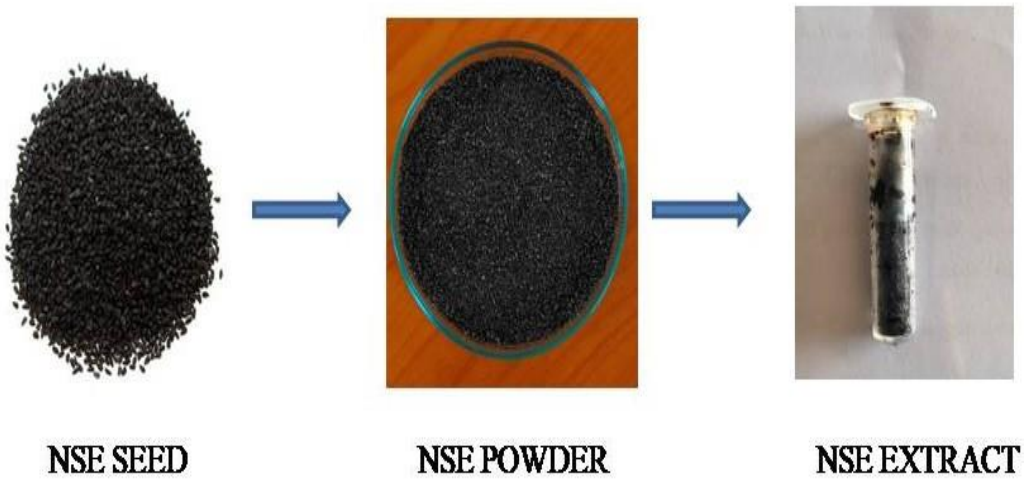

Fig.3.1 Ultrasound assisted Extraction from NSE

3.3. High performance liquid chromatography

Chromatography is a method of separating things into their constituents based on their molecular structure and makeup. A fixed phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas) are required. The mobile phase moves through the stationary phase, carrying the mixture's components with it. Sample components having greater interactions with the stationary phase travel through the column more slowly than those with weaker interactions. The separation of separate components is caused by this disparity in rates. Chromatographic separations can be done using a number of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography), and liquids (liquid chromatography) (liquid chromatography).

Principle

Figure 3.4 depicts high performance liquid chromatography (HPLC), which is essentially a more advanced version of column liquid chromatography. Instead of allowing a solvent to flow naturally through a column, it is pushed through at high pressures of up to 400 atmospheres. This speeds things up considerably. The underlying premise of all

chromatographic separations, including HPLC, is the separation of a sample into its constituent parts due to differences in the relative affinities of distinct molecules for the mobile phase and stationary phase employed in the separation.

Types of HPLC

The following HPLC versions exist, based on the phase system (stationary) in the process.

1. Hplc in normal phase

This approach isolates analyses based on polarity. NP-HPLC employs polar stationary phase and non-polar mobile phase. As a result, the stationary phase is often silica, while the mobile phases include hexane, methylene chloride, chloroform, diethyl ether, and combinations of these. Polar samples are thus kept on the polar surface of the column packing for a longer period of time than less polar materials.

2. Reverse phase hplc

The stationary phase is nonpolar (hydrophobic), whereas the mobile phase is a polar liquid, such as water-methanol or acetonitrile mixtures. It operates on the basis of hydrophobic interactions, hence the less polar the substance, the longer it will be maintained.

3. Size exclusion hplc

The column is loaded with material with exact pore diameters, and the particles are sorted based on their molecular size. Larger molecules are washed through the column quickly, whereas smaller molecules enter the porous packing particles and elute slowly.

4. Ion-exchange hplc

The stationary phase has an ionically charged surface that is charged in the opposite direction as the sample ions. This approach is nearly often employed with ionic or ionizable materials. The higher the charge on the sample, the more it will be attracted to the ionic surface and hence take longer to elute. The mobile phase is an aqueous buffer in which elution time is controlled by both pH and ionic strength.

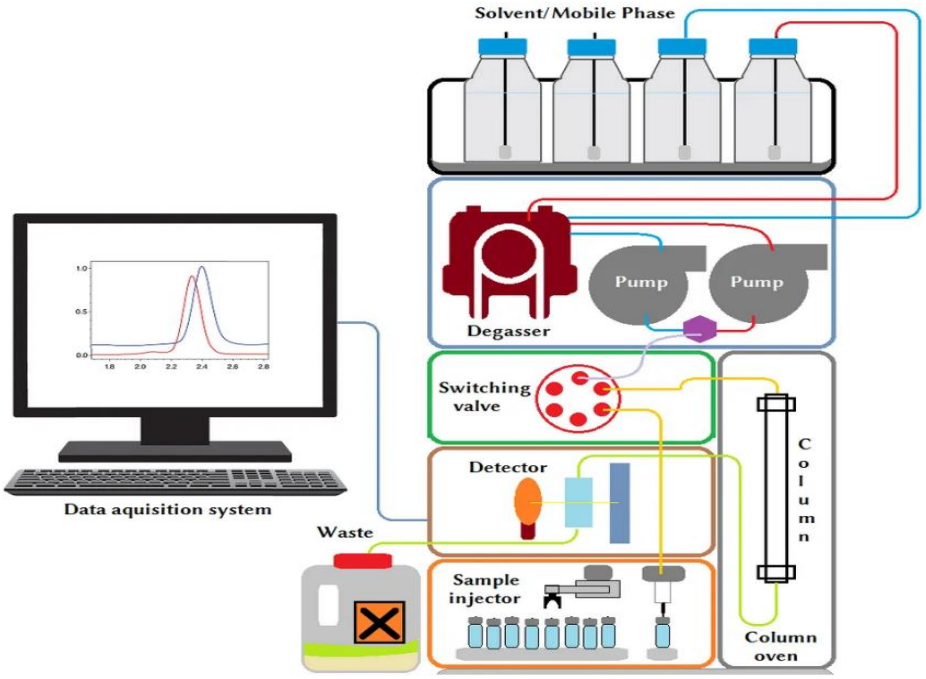

Fig3.2 HPLC (High Performance Liquid Chromatography)

Working

HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occur is shown in fig 3.2

1. Solvent resorvoir

Mobile phase contents are contained in a glass resorvoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.

2. Pump

A pump aspirates the mobile phase from the solvent resorvoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 KPa can be generated.

3. Sample injector

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

4. Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 µm. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

5. Detector

The HPLC detectors, located at the end of the column detect the analytes as they elute from the chromatographic column. Commonly used detectors are UV visible spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

6. Data collection devices

Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret [43].

3.4 Analysis of black cumin seed extract by Hplc

The isocratic mobile phase utilized was composed of water: methanol: 2-propanol (50:45:5% v/ v), and was filtered through a 0.45 mm Millipore filter and desecrated before use. Analyses were performed at room temperature. UV monitoring of the eluted solutes was carried out at 254 nm for TQ. The extracts / compounds were dissolved in an appropriate volume of HPLC grade methanol and 20µL of the sample was injected into the reverse phase C8 column of the HPLC (Aglient technology). Detection was carried out using a photodiode array detector (Prominence SPD-M20A). Chromatographic data were obtained and processed with the software of LC-WorkStation VP^{TM}.

HPLC only Maximum pressure: Low to mid (400 to 600 bar)

Dispersion volume: High to low

3.5 Antibacterial activity

A well diffusion method was used for determination of the antibacterial activities of the *NSE*, and TQ the following steps were carried out:

The antibacterial activity of TQ and *NSE* is performed by Agar well diffusion method using Muller-Hinton Agar as growth media. The bacterial strains namely *Staphylococcus aureus (Gram-positive) and Pseudomonas aeruginosa (Gram-negative)* are used in this study. Sterile agar plates were inoculated and 20 µl of TQ and *NSE* were added separately into the wells bored in the agar medium. Then the plates were incubated at 37º C for 24hrs.A well loaded with ciprofloxacin served as positive control and was maintained on each plate, and for the assessment of the results, pictures of the Petri plates were taken at the end of each experiment and the inhibition zones were measured and recorded.[44]

3.6 2, 2-diphenyl-1-picrylhydrazyl (dpph) radical scavenging assay

The antioxidant activity of the TQ was analyzed by (2, 2 –diphenyl-1-picrylhydrazyl) radical scavenging method. The $DPPH^{\bullet}$ radical exhibits purple in colour in methanol and show a strong absorption band maximum at 517 nm. The concentrations of TQ, ranging from 0.1×10^{-6} Mto 1.0×10^{-6} M, were mixed with 500µL of DPPH (0.3mM). The solutions were mixed thoroughly and incubated in the dark for 30 minutes at room temperature.The $DPPH^{\bullet}$ in methanol was used as a control. The antioxidant activity of all the samples was estimated based on the percentage of DPPH radical scavenged, according to the following equation:

$$\%\ \text{of DPPH radical scavenging} = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] \times 100 \longrightarrow (1)$$

Where $A_{control}$ is the absorbance of DPPH radical in the absence of antioxidants and A_{sample} is the absorption of DPPH radical with TQ. [45]

3.7 Theoretical methods

A theoretical model or method is a way to model a system using a specific set of approximations. These approximations are combined with a calculation algorithm and are applied to atomic orbitals, defined by the basis set, in order to compute molecular orbitals and energy. In general the methods can be separated into four main types. They are

1. Ab initio methods
2. Density functional methods
3. Molecular Mechanics methods

4. Semi empirical methods

3.7.1 Ab initio methods

The Ab initio means “from the beginning” or “from the first” in Latin. It is the first principle for polyatomic molecule. The most common type of ab initio calculation is called Hartree fock calculation (HF), in which the primary approximation called central field approximation. The electronic structure methods are based upon Quantum Mechanics and therefore provide the most accurate and consistent predictions for chemical systems. Ab initio calculations use the true Molecular Hamiltonian and not the empirical data in the calculation except for the fundamental constants of nature such as the mass of the electron, planck’s constant etc that are required for the arrival numerical prediction .

The main uses of the ab initio method are calculating molecular geometries, energies, vibrational frequencies, spectra, ionization potential, electron affinities. The good side of Ab initio methods is that they eventually converge to the exact solution, once all of the approximation are made sufficiently small in magnitude. The bad side of Ab initio methods is that they are expensive. These methods often take enormous amounts of computer CPU time, memory and disk space. Ab initio calculations give very good qualitative results and can give increasingly accurate results as the molecules become smaller.

Advantages of ab initio method

- Ab initio method is useful for broad range of systems.
- It does not depend on experimental data.
- It is capable of calculating transition states and excited states.

Ab initio method is best for:

- Small systems (ten of atoms).
- Systems involving electronic transition.
- Molecules without available experimental data.
- Systems requiring rigorous accuracy.

Hartree-fock method

The most rigorous method for the determination of orbitals in many electron atoms is provided by the Hartree Fock theory. The Hartree method had been formulated in 1928 and approximated an N-electron wave function as a simple product of N one electron functions

$$\Phi\ (1)\ \phi\ (2)\ldots\ldots\ldots\ \phi\ (N) \longrightarrow (2)$$

The electron spin have not been included in this theory. The Hartree wave function does not obey the antisymmetry principle. A couple of years later Fock modified the Hartree theory by including electron spin and resulting formulation is known as the Hartree Fock theory. The aim of the HF theory is to determine the one electron function or orbitals which minimize the energy expectation value of the Slater's determinant (antisymmetrized product) constructed from the orbitals. Therefore, a Hartree-fock calculation is an ab initio (as is an SCF) calculation that gives only an approximation to the Hartree-fock wave function.

Limitation of the Hf method

Hartree-fock approximation solves equations of the behaviour of each electron in the average field on the remaining electrons. Unfortunately electrons respond to each other in an instantaneous manner through the coulomb's law. The motion of each pair of electron is correlated and this electron correlation is neglected in the HF model which forms its drawback. The correlation energy is defined as

$$E_{corr} = E_{exact} - E_{HF} \longrightarrow (2)$$

i.e the difference between the (non-relativistic) exact energy and the HF limit energy. Great deal of modern work in the field of electronic structure calculation is aimed at taking electron correlation into account. This method also has another disadvantage that it does not give pure spin state.

3.7.2 Density functional theory (DFT)

Density Functional theory are often considered to be ab initio methods for determining the molecular electronic structure, even though many of the most common functional use parameters derived from empirical data, or from more complex calculations. This means that they could also be called semi-empirical methods. In DFT, the total energy is expressed in terms of the total electron density rather than the wave function. In this type of

calculations, there is an approximate Hamiltonian and an approximate expression for the total electron density. DFT methods can be very accurate for little computational cost. The drawback is that unlike ab initio methods, there is no systematic way to improve the methods by improving the forms of the functional. Density functional theory based methods are ultimately derived from Quantum Mechanics research, especially from the Thomas-Fermi-Dirac model in 1920's and from the Slater's fundamentals work in Quantum Chemistry in the year 1950's. The DFT approach is based upon a strategy of modelling electron correlation of the electron density.

Density functional theory are proposed solely in terms of the one electron density and based upon the Hohenberg-Kohn vibrational principle which provide no general procedure for accurately calculating relatively small energy difference such as excitation energies, ionisation potentials and electron affinities. A power full method inspired by the Hohenberg-Kohn vibrational principle has been used with great success in the calculation of such quantities. Following on the work of Khon and Sham, the approximate functional employed by current DFT methods partition the electronic energy into several terms:

$$E = E^T + E^V + E^J + E^{XC} \longrightarrow (3)$$

Where, E^T = Kinetic energy terms (arising from the motion of electrons)

E^V = The potential energy of the nuclear- electron attraction and of the repulsion between pairs of nuclei.

E^J = Electron-electron repulsion term (i.e. coulomb self-interaction of the electron density)

E^{XC} = Exchange correlation term and includes the remaining part of the electron-electron interactions.

Kohn sham equation

In order to obtain an electron density from DFT, some assumptions are made that are analogous to those made in solving the Schrodinger equation. For the Schrodinger equation, LCAO-MO approximation used to obtain a trial wave function. In DFT, the similar approximation for the trial electron density,

$$\rho_t(x,y,z) = \sum_i \left|\Phi_i^{ks}(x,y,z)\right|^2 \longrightarrow (4)$$

Φ_i^{ks} , are Kohn Sham orbitals, which are similar to the molecular orbitals that appear in the approach for solving the Schrodinger equation approximately using the Hartree- Fock method. Each Kohn- Sham orbitals holds two electrons and can be represented using a linear combination of atomic orbital basis function.

$$\Phi_i^{ks}(x,y,z) = \sum_{\mu=0}^{ks} c_{\mu i} f_\mu(x,y,z) \quad \longrightarrow (5)$$

In this equation, $c_{\mu i}$ corresponds to numerical coefficients and the functions $f_\mu(x,y,z)$ are the atomic orbital basis function. The coefficients $c_{\mu i}$ are obtained by solving a set of equations called the Kohn-Sham equations. These are analogous to the Hartree- Fock equations employed when obtaining a solution of the Schrodinger equation.[46]

Advantages of DFT method

- Density functional theory is computationally very efficient.
- DFT is conceptually simple.
- DFT can be easily combined with Molecular Dynamics of the nuclei.
- In the basis set limit, the Hartree-fock equations would not give the correct energy, but the Khon-Sham equations would correct energy by knowing the exact exchange correlation energy functional.
- In wave function theory, Hartree-fock level results are improved by using perturbation or configuration interaction treatments of electron correlation, but in DFT theory there is as yet no systematic way of improving the exchange-correlation energy functional.
- The solution to the Hartree-fock equation may be viewed as exact solution to the approximate description, while the solutions to the kohn-sham equations are approximation to an exact description.

Application of DFT

- DFT is a general- purpose of computational method,, and it can be applied to most of the systems.
- Like all the computational methods, DFT methods are more useful for some types of calculations than others.
- Some DFT methods are specifically designed for specific applications, such as the MPW1k hybrid method, designed for determination of kinetic problems.

- Application of modern DFT calculations has been extended from small molecules for testing the accuracy to transition metal complexes.[47]

3.7.3 Molecular mechanics

In many cases, large molecular system can be modelled successfully while avoiding Quantum Mechanical calculation entirely. For example Molecular Mechanics simulations use a single classical expression for the energy of a compound for instance the harmonic oscillator. All constants appearing in the equations must be obtained beforehand from experimental data or ab initio calculations. The database of compounds used for parameterization is crucial to the success of Molecular Mechanics calculations. A force field parameterized against a specific class of molecules, for instance proteins, would be expected to only have any relevance when describing other molecules of the same class.

Advantages of molecular mechanics

- It requires less of a computer than Quantum Mechanical methods.
- It can be used for molecules as large as enzymes.
- An important advantage of Molecular Mechanic method is their efficiency.

3.7.4 Semi-empirical models

The evolution of Molecular Orbital (MO) methods has resulted in two main methods, ab initio and semi empirical. Semi empirical methods are simplified versions of Hartree-fock theory. The semi empirical calculations are much faster than the ab initio calculations. Such methods are currently very slow and routine application at any reasonable degree of accuracy to systems of even a few tens of atoms is still not practical. In contrast, semi empirical methods employ empirically determined parameters and less attractive to the theoretical purist. Unlike ab initio methods, the accuracy of any empirical method is limited to the accuracy of the experimental data used in obtaining the parameters. However, semi-empirical methods are fast enough and accurate enough for routine application to quite large systems.

Semi empirical methods are based on three approximation, they are

- The elimination of core electrons from the calculation.
- The use of minimum number of basis sets.
- The reduction of the number of two electron integrals.

Semi empirical calculations have been most successful in the description of organic chemistry, where only a few elements are used extensively and molecules are of moderate size. However, semi empirical methods were also applied to solid and nanostructure but with different parameterization.

Advantages of semi empirical method

- Less demanding computationally than ab initio method.
- Capable of calculating transition state and excited state.

Semi empirical methods are best for:

- Medium sized systems.
- Systems involving electronic transition.[48]

3.8 Basis functions

The calculation of molecular integrals is the most cumbersome step in the calculation of electronic wave function. There are numerical difficulties which depend on the choice of the basis set of function $\{\Phi_v\}$. In principle, for high frequency, a complete basis set should be used. In practice a finite basis set of reasonable dimension is used. The functions are generally so chosen as to create least difficulty in integration. [49]

3.8.1 Basis sets

A basis set is a mathematical description of the orbital's with in a molecular system used to perform Quantum Chemical calculations. Larger the basis set, more accurately the approximate orbitals, by imposing a fewer restrictions to the electron in space.[50]

3.8.2 Classification of basis sets

The basis sets can be broadly classified in to the following types.

- **Minimal basis sets (sto-3g)**

In minimal basis set each AO is represented by an STO which in turn is a linear combination of N GTF'S (Gaussian type function). Once the coefficients of expansion and the Gaussian orbital exponents are determined and are fixed throughout the calculations. The most commonly used minimal basis sets is STO-3G. A Gaussian basis set is often denoted by

the notation (A) (B) or (A/B), where A is a listing of the primitive Gaussian and B is a listing of the contracted Gaussians. The second notation is more convenient to use.

STO-3G basis set for H and LI are (3s) [1s] or (3s/1s) and (6s3p) [2s1p] respectively. The integers should not be confused with the principal quantum numbers.

Commonly used minimal basis sets are

- STO-3G
- STO-4G
- STO-6G
- STO-3G*

➢ **Extended basis sets**

There are several types of extended basis sets:

- Double zeta and triple zeta basis sets
- Split- valence basis sets
- Polarized basis sets
- Diffuse sets

➢ **Double-zeta and triple-zeta basis sets**

The double zeta valance basis sets form molecular orbitals from the linear combinations of two sets of functions for each atomic valence orbital. Similarly, triple split valence basis sets such as 6-311G, use three sets of contracted functions for each valence orbital type.

➢ **Split valence basis sets**

Split valence basis set is one of way to increase the size of a basis set is to take more basis functions per atom. Split valence basis sets, such as 3-21G and 6-31G basis sets, have two or more size of basis function for each valence orbital. It has only the valence part of the basis set is doubled.

➢ **Polarised basis set**

Split valence basis set could be improved by adding orbitals with different shapes. Polarized basis sets add orbital's with angular momentums going beyond of requirement for

the proper description of the ground state of each atom at the HF level. For example, polarized basis sets add d-functions to carbon atoms and some of them add p-function to a hydrogen atom.

Examples for polarized basis sets are

- 6-31G (d)
- 6-311G (d,p)

Diffuse basis set

Basis sets with additional diffuse functions are large by size versions of and s and p-type split valence basis sets. Diffuse orbitals occupy a larger region of space. Basis sets with diffuse functions are important for systems where electrons may be far from the nucleus. One example for diffuse basis function is the 6-311+G (d, p) basis sets.[51]

3.9 Optimized geometrical parameters

3.9.1 Bond length and bond angle

The distance between centers of bonded atoms are called bond lengths, or bond distances. Bond length vary depending on many factors, in general they are very consistent. The bonds orders, the bond length, and of the same order for the same pair of atoms in various molecules are consistent. The bond length ranges from the shortest of 74pm for H-H to 200pm for long atoms, and the bond energies depends on bond order and lengths. The average angle between the orbital's of the central atom containing the bonding electrons pair in the molecules is known as bond angle between the atoms. The unit of bond angle is either degree or minute or second. The gives the distribution of orbital's around the central atom in a molecule. Therefore bond angle determines the shape of a molecule.

3.9.2 Bond dissociation enthalpy (BDE)

Hydrogen atom transfer (HAT) is a major mechanisms by which phenolic antioxidant exert its anti oxidative capacity, H atom directly transfers from antioxidant to active free radical to break chain react. The mechanism occurs when an antioxidant compound quenches free radical species by donating hydrogen atom. The reactivity of an antioxidant can be estimated by the C-H bond. BDE value calculated according to the formula [52]

$$\mathrm{BDE} = \mathrm{H}(\mathrm{Ar\,O^{\cdot}}) + \mathrm{H}(\mathrm{H^{\cdot}}) - \mathrm{H}(\mathrm{ArOH}) \rightarrow (6)$$

Where, H (Ar O·) = Enthalpies of the phenoxy radical

H (H·) = Enthalpy of hydrogen atom

H (ArOH) = Enthalpy of neutral molecule

In HAT, the reactivity of an ArOH can be estimated by calculating the C-H bond dissociation enthalpy (BDE), where the lower BDE value the higher the expected antioxidant activity. In order to calculate BDE value enthalpies of H-atom, in gas phase and solvents are needed. The gas phase enthalpy of a hydrogen atom is -0.01024 hartree [65].

3.10 Topological analysis

Topological analysis, namely hydrogen bond energy (EHB), electron density (ρ), Laplacian of electron density (∇2ρ) and ellipticity (ε) values at bond critical point (BCP) were calculated using Bader's quantum theory of atoms in molecules between TQ and DPPH• complex [36]. Density of states, ELF and LOL are plotted for the complexes using Multiwfn 3.4.1 [37]. The charge transfer between TQ and DPPH• is investigated using NBO analysis. The stabilization energy E (2) is calculated from second order perturbation theory associated with electron delocalization of don or (i) → acceptor (j),

$$E(2) = \Delta Eij = qi\frac{F2(i,j)}{\varepsilon i - \varepsilon j}$$

where, qi is the donor orbital occupancy, εi and εj are diagonal elements (orbital energies) and F(i,j) is the off-diagonal Fock matrix element.[53]

3.9 Global descriptors

The E_{HOMO} energy of a molecule shows the electron-donating ability, whereas E_{LUMO} characterizes electron-accepting ability. Ionization potential (IP) and electron affinity (EA) are also roughly accompanying with E_{HOMO} and E_{LUMO} according to Janak's Theorem [54].

$IP = -E_{HOMO}$

$EA = -E_{LUMO}$

Hardness (η) is defined as resisting power toward the polarization of electron cloud of a chemical species [25].

Hardness; $\eta = \frac{IP-EA}{2}$

or Hardness; $\eta = \frac{E_{LUMO} - E_{HOMO}}{2}$

Softness is just a reciprocal of the hardness [26].

Softness; $\sigma = \frac{1}{\eta}$

Electronegativity (χ) can be described as the ability of an atom to have more attraction to covalently bonded shared electrons toward itself. It is computed as follows:

Electronegativity, $X = \frac{-(E_{HOMO} + E_{LUMO})}{2}$

3.11 Molecular docking

The monomer BSA crystal structure was taken from the Protein Databank (PDB ID: 3V03), and the flavanols (TQ) were used for molecular docking studies. Using the Del Water Obj and DelMol tools, the protein is pre-processed by eliminating the water molecules and co-crystallized flavanols from the coordinate system. The polar hydrogen atoms were then added to the preprocessed protein according to fundamental chemistry principles and the bond sequences were altered using the AddHyd command. Almost 10 independent docking runs were performed, and minimum energy conformers were saved for each complex. AutoDock Vina performed the computation of grid maps and grouping of dock results automatically. LigPlot+ is applied to find the configuration of hydrophobic interaction of the amino acid residues of BSA with TQ [55]

RESULTS AND DISCUSSION

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

The seed of Nigella sativa L. is one of the herbal medicines that has been widely used to protect the cell membrane from oxidative damage. Antioxidants present in *Nigella sativa* seed (NSE) are scavengers of free radicals and their antioxidant capacities is mainly depending on their ability to scavenge free radicals, and are widely used to suppress the oxidative damage, and are having high biological functionality that includes anti-inflammatory, antimicrobial, antiviral and anticancer activities. The extraction from *Nigella sativa* L.(NSE) consists of active components namely thymoquinone (TQ), thymohydroquinone, dithymoquinone, p-cymene, carvacrol, 4-terpineol, t-anethole, sesquiterpene longifolene, α-pinene, and thymol. The most effective compound found in NSE is TQ. The present study is to identify TQ in NSE by High Performance Liquid Chromatography (HPLC**)**.The antibacterial activity of NSE and TQ was examined using gram positive and negative bacteria namely *S.aureus* and *P.aeruginosa* respectively. The antioxidant activity of thymoquinone was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$**)** assay. TQ is a natural compound present in black cumin which possesses potent antioxidant activity without having any phenolic hydroxyl group which is responsible for antioxidant activity. The structures of TQ and DPPH$^{\bullet}$ were optimized and forms the complex TQ-DPPH$^{\bullet}$ using DFT with the B3LYP/6-311+G (d, p) method. Interaction Energy, Quantum theory of atoms in molecules (QTAIM), natural bond orbital (NBO) and topological analysis were predicated for the complex TQ-DPPH$^{\bullet}$. Global descriptors such as the electron affinity, ionization potential, electronegativity, softness, chemical hardness, chemical potential and energies of E_{HOMO} (Highest Occupied Molecular Orbital), E_{LUMO} (Lowest Unoccupied Molecular Orbital) and the energy gap were calculated by using same method. All calculations were carried out by using the Gaussian 09.The presence of hydrogen bond and the hydrophobic bonds were also studied using the Molecular docking method.

4.2 Experimental study

4.2.1 Hplc analysis

The NSE is dissolved in the volume of HPLC grade water: methanol:2-propanol (50:45:5, v/v) ratio and 20µl of the extract is injected into the reverse phase C8 column of the HPLC. Chromatographic data are obtained using LC workstation VP^{TM} 6.14.Figures 4.1. (a) and 4.1. (b) Illustrate the retention period of the peak found for NSE and TQ. The obtained retention time of NSE is compared with retention time of standard TQ. The retention time obtained in NSE was 14.69 min coincides with retention time of TQ 14.76 min. This confirms the presence of TQ in the NSE. [56]

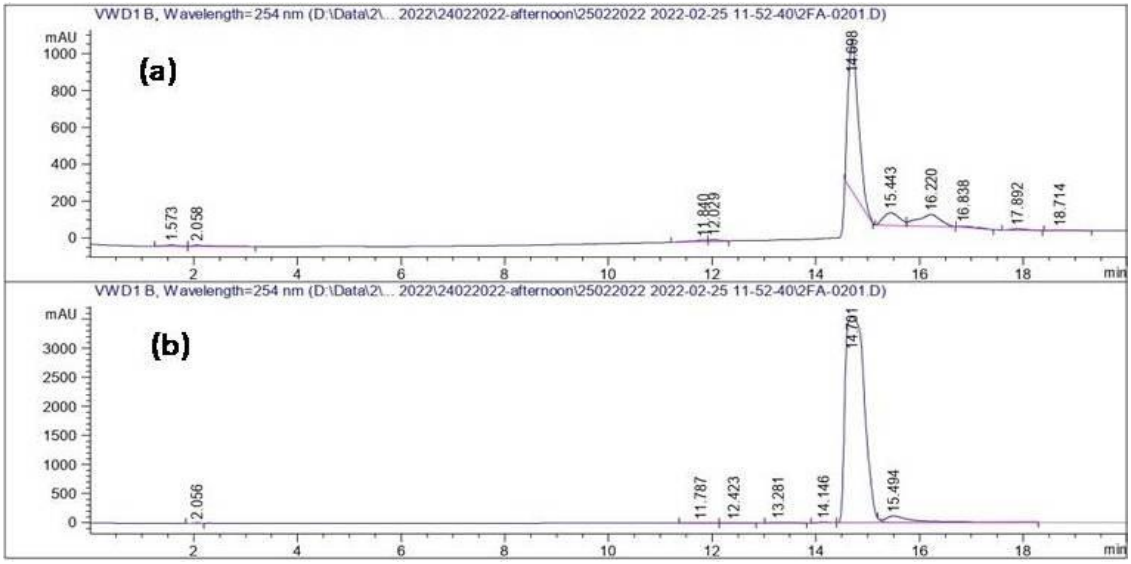

Fig.4.1. (a) and 4.1.(b) HPLC Chromatogram for NSE and standard Thymoquinone

4.2.2 Antibacterial activity

The antibacterial activity is estimated by well diffusion method against *Staphylococcus aureus (S.aureus)* (Gram-positive bacteria) and *Pseudomonas aeruginosa (P.aeruginosa)* (Gram-negative bacteria) for the NSE and TQ and is shown in Figure 4.2. The diameter of the inhibition zones is measured and tabulated in Table 4.1 TQ showed 30 mm wide zone of inhibition against *S.aureus* (Figure.4.2.a) and 45 mm zone of inhibition against *P.aeruginosa*. TQ exhibited antibacterial activity against *S. aureus* and *E.coli* (Figure.4.2.b) with the zone of inhibition of 31 mm and 42 mm respectively. NSE showed strong antibacterial properties with TQ as major phytochemical involved. [57]

Table 4.1 Zone of inhibition of *Staphylococcus aureus (S.aureus)* and *Pseudomonas aeruginosa (P.aeruginosa*) bacteria using TQ and NSE

Compound	*S. aureus* (mm)	*P. aeruginosa* (mm)
TQ	30	45
NSE	31	42

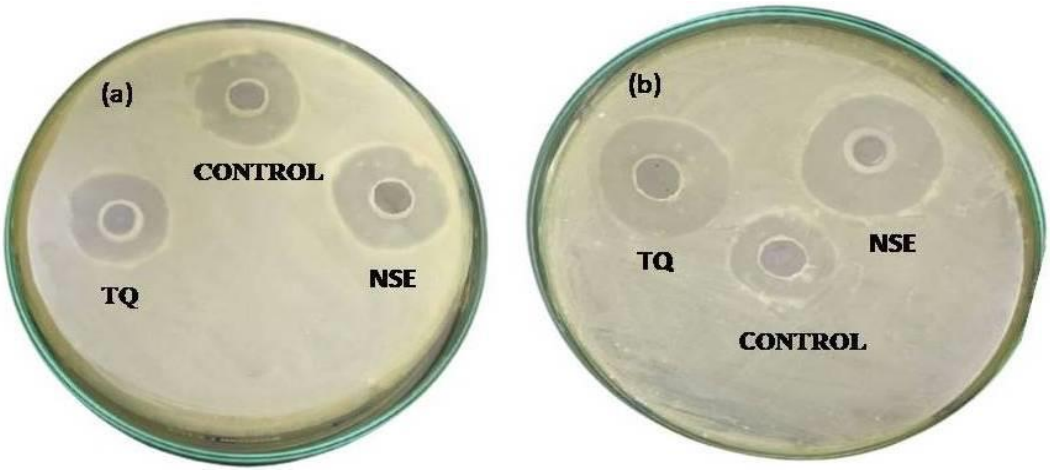

Fig.4.2 Antibacterial activity with zone of inhibition of TQ and NSE against *Staphylococcus aureus (S.aureus)* (4.2.a) and *Pseudomonas aeruginosa(P.aeruginosa*)(4.2.b) respectively

4.2.3 Dpph radical scavenging activity

DPPH$^{\bullet}$ assay was performed to analyze the antioxidant activity of TQ. It is well known that DPPH$^{\bullet}$ is a stable free radical at room temperature, it becomes stable neutral molecule after accepting a hydrogen atom or electron from antioxidants. The free radical of DPPH$^{\bullet}$ exhibits purple in color in methanol and its absorbance was recorded in the range from 200 to 800 nm. The scavenging activity of TQ was investigated with the concentration from 0.1×10^{-6} Mto1.0×10^{-6} M. The optical absorbance value at 517 nm decreases while increasing the concentration of TQ. In addition to that, the decolorization of DPPH solutions from purple to yellow color is observed. This decolorization of DPPH$^{\bullet}$ confirms the

scavenging ability of TQ via hydrogen atom or electron transfer[39]. Hence, the scavenged DPPH radical is transformed to stable 1,1diphenyl picryl hydrazine molecule. Further, the percentage of scavenging was calculated from Equation (1) and is summarized in Table 4.2 to confirm the scavenging ability of TQ [58].

Table 4.2
Percentage of scavenging and with respect to concentration of TQ against DPPH radical

Concentration(10^{-6} M)	Scavenging ability of TQ in %
0.1	7.86
0.2	12.9
0.3	21.2
0.4	32.3
0.5	44.0
0.6	56.4
0.7	60.9
0.8	71.3
0.9	75.1
1.0	80.5

The half maximal inhibitory concentration (IC_{50}) values were determined from the plot of the percentage of inhibition versus the sample concentration by linear regression (Fig.4.3) which is found to be 0.59 x 10^{-6} M for TQ respectively.

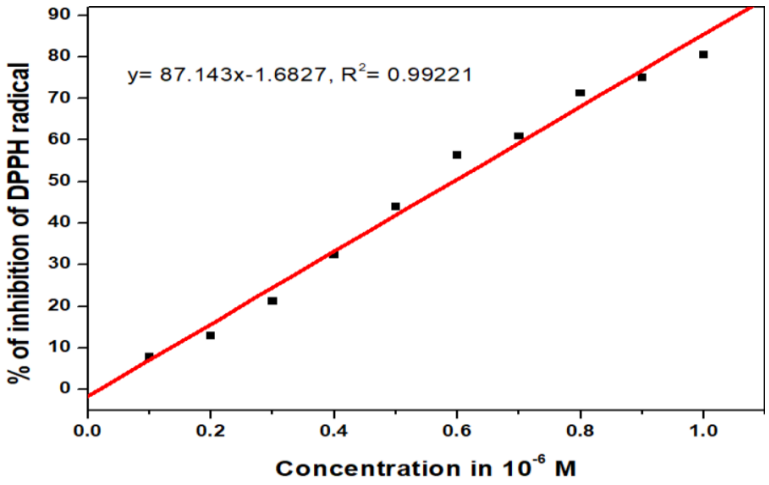

Fig.4.3 Percentage of inhibition of DPPH radical with concentration of TQ from 0.1to 1.0 × 10-6 M

It is important to note that TQ have superior scavenging activity through HAT or SET mechanisms. Therefore, TQ can be used as a scavenger against DPPH radical.

4.3 Theoretical study

4.3.1. Optimized structural properties

The optimized structure of isolated TQ, DPPH$^{\bullet}$ and the complex TQ-DPPH$^{\bullet}$ are shown in Figs.4.4 (a), (b), (c) respectively. The BDE of C-H group is a useful descriptor in structure activity analysis of antioxidants .The hydrogen bond is abstracted from each site namely C12, C6, and C5. From the hydrogen abstracted site, the BDE value found to be 82.44, 98.90 and 98.90 kcal/mol, respectively.C12 is the most preferential active site for hydrogen atom donation in TQ compound. Hence, the active site C12 has been chosen as the preferential site for the interaction with DPPH radical. Therefore, the TQ-DPPH$^{\bullet}$ complexes have been formed in the vicinity of nitrogen atom (N32) of DPPH$^{\bullet}$ and the hydroxyl group at C12 position of TQ through intermolecular hydrogen bond C–H···N. The selected bond lengths of TQ-DPPH$^{\bullet}$ are summarized in Table 4.3. Due to the interaction of TQ with DPPH$^{\bullet}$, the C-H at quinone moiety, N-N and N-C at picryl ring are slightly altered, while the remaining bond length of TQ and phenyl ring of DPPH$^{\bullet}$ remains unchanged.

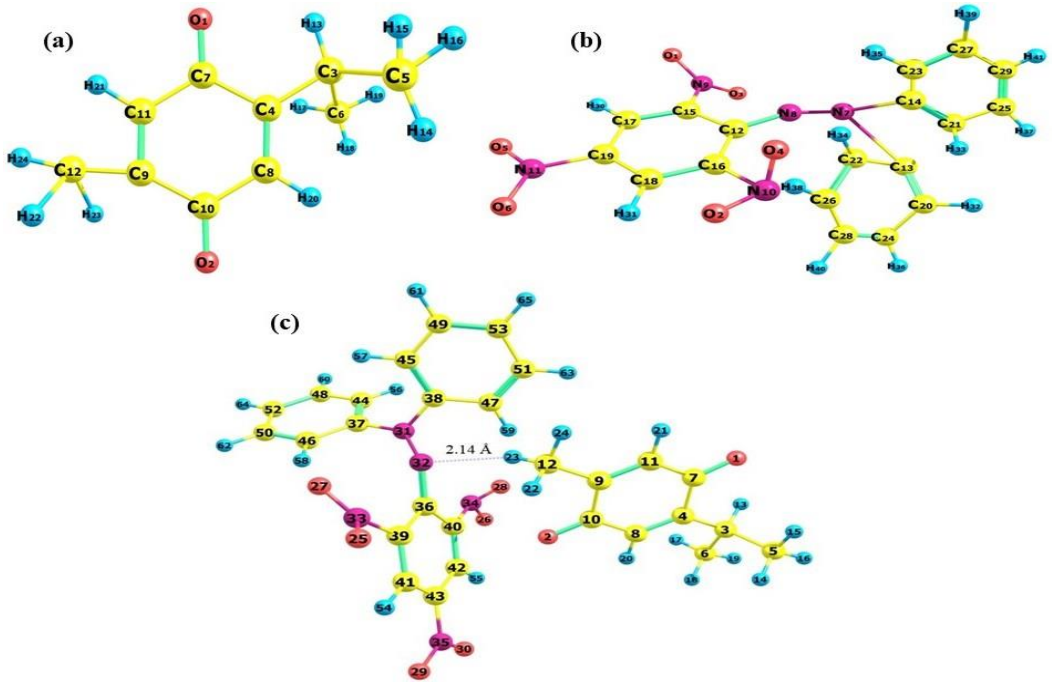

Fig4.4. (a) The optimized structures of TQ (b) DPPH$^{\bullet}$ and (c) TQ- DPPH$^{\bullet}$ complex

The bond length of C-H and N-C exhibits elongation of about 0.01Å in TQ-DPPH•. Based on the Lewis acid-base concept, the C12-H23 bond in TQ act as a hydrogen donor, and the electronegative nitrogen atom N32 of TQ-DPPH• act as a hydrogen acceptor. The hydrogen atoms H23 of TQ is involved in strong hydrogen bond with nitrogen (N32) of DPPH radical with bond length of 2.14 Å, and bond angle of 177º.The computed shorter bond length and larger bond angle for TQ suggest that it forms a stronger hydrogen bond interaction with DPPH•[59].

Table.4.3.Bond length (Å) of TQ, DPPH• and TQ-DPPH• complex

Parameters	Bond length(Å)
C12-H23….N32	2.00(2.14) •
C3-H13	1.08(1.09) •
C3-H5	1.53(1.54) •
C4-C3	1.51(1.50) •
C6-H17	1.08(1.09)•
C6-H19	1.09(1.08) •
C12-H24	1.08(1.09) •
C12-C9	1.49(1.51) •
N31-C38	1.41(1.42) •
C37-C46	1.38(1.39) •
C12-H23	1.09(1.08) •
C12-H22	1.09(1.08) •
C5-H15	1.08(1.08) •
C5-H14	1.09(1.09) •
C5-H16	1.09(1.09) •
C6-H18	1.09(1.09) •
C9-C10	1.49(1.49) •
N31-C37	1.43(1.43) •
N32-C36	1.39(1.39) •
C37-C44	1.39(1.39) •
C44-H56	1.08(1.08) •
C48-H60	1.08(1.08) •
C47-H89	1.07(1.07) •
C53-H65	1.08(1.08) •
C45-H57	1.08(1.07) •

***The number in parenthesis belongs to bond length of monomer TQ and DPPH•**

4.3.2 Interaction energy of TQ- DPPH$^{\bullet}$

In the contest of scavenging of free radicals, the analysis of interaction energy can provide information about binding between scavenger and radical. In the present study, ΔE of TQ and DPPH$^{\bullet}$ is calculated using counterpoise method with BSSE correction. The negative sign of interaction energy indicates the formation of TQ-DPPH$^{\bullet}$ complexes which is thermodynamically favorable. The TQ-DPPH $^{\bullet}$have interaction energy of -6.43 kcal/mol, respectively. The interaction energy could correlate to the observed hydrogen bond strength.

4.3.3 Quantum theory of Atoms in molecules (QTAIM) and NBO analysis

The presence of intermolecular hydrogen can be analyzed during Bader's quantum theory of atoms in molecule. Based on this theory the existence of bond path between hydrogen bond donor and hydrogen acceptor is characterized through various parameters related to electron density at bond critical point. From this analysis, electron density (ρ(r)), Laplacian of electron density ($\nabla^2\rho(r)$), total energy electron density (H(r)), ratio of kinetic (G(r)) and potential (V(r)) energy electron density, -G(r)/V(r) and ellipticity (ε) values at BCP are calculated and tabulated [60]inTable.4.4

Table 4.4

Topological parameters of the C–H…N BCPs of the TQ interacted with DPPH$^{\bullet}$- Electron density (ρ), Laplacian of electron density ($\nabla^2\rho$), ellipticity (ε), kinetic energy density(G(r)), potential energy density (V(r)),-G(r)/V(r) and total energy electron Density (H(r))(all the values in a.u.)

Complex	Bonding type	ρ (r) (a.u)	$\nabla^2\rho(r)$ (a.u)	E (a.u)	G(r)(a.u)	V(r)(a.u)	-G(r)/V(r) (no unit)	H(r)(a.u)
TQ-DPPH$^{\bullet}$	C12-H23…N32	0.0278	0.0856	0.0462	0.0203	-0.0191	1.0596	0.0011

In TQ-DPPH$^{\bullet}$ complex, the value of ρ(r) and $\nabla^2\rho(r)$ are found to be 0.0278and 0.0856a.u. Therefore, the interaction between TQ and DPPH$^{\bullet}$ is electrostatic in nature. Additionally, from Table 4.4,it is found that the H(r)> 0and the ratio of –G(r)/V(r) is greater than 1 for the complex, which indicates that the interaction between TQ and DPPH$^{\bullet}$ is electrostatic in nature. [61] Ellipticity (ε) is defined as $\varepsilon = \frac{\lambda_1}{\lambda_2}$-1 where,$\lambda_1$ and λ_2 is negative eigen values of the Hessian of the electron density at the BCP. The positive value of ellipticity is observed in TQ-DPPH$^{\bullet}$ complex which shows that the electron density distribution at the BCP is

anisotropic for the complex.The hydrogen bond energy (E_{HB}) is calculated using the method developed by Espinosa et al. [34]. The E_{HB} is calculated using the formula, $E_{HB} = \frac{1}{2V(r)}$,where V(r) is the potential electron density at BCP. The value of E_{HB} is -6.00 kcal/mol.The negative sign of E_{HB}indicates that the hydrogen bond formation between TQ and DPPH$^{\bullet}$ is thermodynamically favored. [62]

NBO analysis is used to study the charge transfer between TQ and DPPH radical through hydrogen bond C-H…N. [63].The second order stabilization energy E(2) is calculated as a measure of the amount of charge transfer between lone pair of the proton acceptor and anti bonding orbital of the proton donor and is listed in Table.4.5.

Table.4.5

Bond stabilization energy E (2) (in kcal/mol) using second-order perturbation theory between lone pair n(y) in the proton acceptor and anti bonding orbital n (σ^*) in the proton donor for TQ-DPPH$^{\bullet}$ complex calculated by the NBO method.

Molecule	Bonding type	n (y)	n (σ^*)	E(2) kcal/mol
TQ- DPPH$^{\bullet}$	C12-H23…..N32	LP(1) N32	σ^* (1) C12-H23	4.15
	C12-H23…..N32	LP(2)N32	σ^* (1) C12-H23	1.88
	C9-C11…..H23	LP(2)C11	σ^*C12-H23	1.09
	C9-C12…..H23	LP(1)H23	σ^*C12-H23	0.38
TQ		σ C $_4$-C $_8$	σ^*O 2 – C 10	10.4
		σ C 9 – C11	σ^*O 1- C 7	10.2
		σ C9 – C11	σ^*O 2 – C 10	8.99
		σ O 1 – C 7	σ^*C 4 – C 8	11.6
		σ O 1 – C 7	σ^*C 9- C11	12.5
DPPH$^{\bullet}$		σ O 25 – O 27	σ^*N 33	4.33
		σ C46 – C50	σ^*C37 – C44	11.06
		σ C46 – C50	σ^*C48- C52	9.25
		σ C47 – C51	σ^*C38 – C45	11.74
		σ C41- C51	σ^*C49 – C53	8.53
		σ C48 – C52	σ^*C37 – C44	10.24
		σ C48 – C52	σ^*C46 – C50	11.06
		LP (2) (O25)	σ^*O27 - N33	9.70

		LP (2) (O25)	σ^{*}N33 – C39	6.29
		LP (2) (O26)	σ^{*}O28 – N34	9.45
		LP (2) (O26)	σ^{*}N34 – C40	6.41
		LP (1) (C36)	σ^{*}C39 – C41	23.2
		LP (1) (N33)	σ^{*}O25 – O27	487.0
		LP (1) (N35)	σ^{*}O29 – O30	466.1

From 4.5 strong delocalization with greater stabilization occurs at LP(1)H23 → σ^{*}(C12-H23) (0.38kcal/mol),σC_4-C→σ^{*}O2–C10(10.4kcal/mol),σC9–C11→σ^{*}O2–C10(8.99kcal/mol),σO1–C7→σ^{*}C4–C8(11.6kcal/mol),σO1–C7→σ^{*}C9-C11(12.5kcal/mol),σC46–C50→σ^{*}C37–C44(11.06kcal/mol), σC47–C51(11.74kcal/mol), σC48–C52→σ^{*}C37–C44(10.24kcal/mol), σC48–C52→σ^{*}C46 – C50(11.06kcal/mol), LP (1) (C36) → σ^{*}C39 – C41(23.2kcal/mol), LP (1) (N33)→σ^{*}O25–O27(487.0kcal/mol), LP (1) (N35) →σ^{*}O2 –O30(466.1kcal/mol) of TQ-DPPH$^{\bullet}$ complex show the presence of intramolecular charge transfer from alone pair of oxygen atom to anti bonding orbital of C-H bond within TQ .During the complex formation, the interaction occurs between lone pair of the hydrogen bond acceptor, nitrogen atom of DPPH$^{\bullet}$ and anti bonding orbital of hydrogen bond donor C-H bond of TQ [64].It is found that the E(2)of the C-H...N bond in TQ-DPPH$^{\bullet}$ complexes is 4.15 and 1.88 kcal/mol, respectively. Thus, the stability of the TQ-DPPH$^{\bullet}$ is attributed to the intermolecular charge transfer through hydrogen bond between TQ and DPPH$^{\bullet}$. Thereby, it is conclusive that hydrogen bond drives the mechanism of antioxidation, as TQ counters a DPPH radical.

4.3.4. Electron localization function and localized orbital locator

The topological analysis of electron localization function and localized orbital locator which were done for TQ-DPPH$^{\bullet}$ complexes are shown in Fig.4.5.

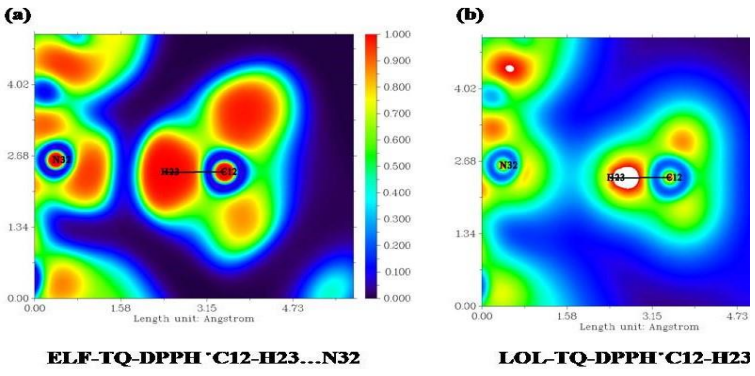

Fig.4.5. (a) Electron location function (ELF) and (b) localized orbital locator (LOL) of C–H…N bond ofthe TQ interacted with DPPH$^{\bullet}$

These maps are useful to understand the localization of electrons at each atom in the complex, through which the type of interaction between the molecules during the complex formation is studied. The scale ranges from 0 to 1.0 is the indicators of color from blue to red for ELF, and 0-0.8 is the scale range for LOL. The large value from 0.5 to 1.0 indicates bonding and non-bonding localized electrons, and the smaller value (< 0.5) indicates the regions where the electrons are likely to be delocalized. The presence of a covalent bond is indicated by a high localization of electrons[65]. As shown in Fig.4.5. a and b, the blue color region with a low value (0.300) of ELF and the cyan colour region (0.280) of LOL are identified at the vicinity of hydrogen atom (H23-TQ) and nitrogen atom (N32) of DPPH$^{\bullet}$ shows electrostatic interactions(non-covalent) between TQ and DPPH$^{\bullet}$[66].

4.3.5 Frontier molecular orbital analysis

The highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of TQ-DPPH$^{\bullet}$ are shown in Fig.4.6.

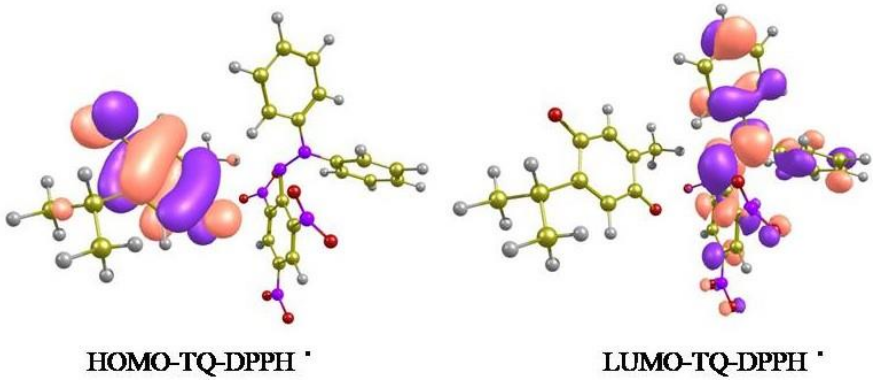

Fig.4.6. (a) Electron location function (ELF) and (b) localized orbital locator (LOL) of C–H…N bond ofthe TQ interacted with DPPH$^{\bullet}$

The HOMO of TQ-DPPH$^{\bullet}$ is mainly delocalized on quinone ring of TQ. The LUMO of TQ-DPPH$^{\bullet}$ is delocalized more on the picryl ring of DPPH$^{\bullet}$. That is, antioxidants TQ act as a nucleophile and DPPH$^{\bullet}$ act as an electrophile. HOMO energy of TQ-DPPH$^{\bullet}$ is −7.29 eV, and LUMO energy is −3.87 eV. The energy gap is found to be -3.41 eV, which shows the stability of the complex TQ-DPPH$^{\bullet}$.[67]

4.3.6 Global descriptors

The global molecular descriptors, such as ionization potential, electron affinity, electronegativity, chemical hardness, chemical potential, and softness, will provide information about chemical reactivity and stability of the complex. The calculated global molecular descriptors for TQ-DPPH$^{\bullet}$ is summarized in Table 4.6. It also provides information about the tendency to release or capture the electron, which is one of the key aspects of the antioxidant potential. It is observed that Ionization potential (IP_V) of TQ-DPPH$^{\bullet}$is-7.29eV, respectively. The electron affinity (EA_V) of TQ-DPPH$^{\bullet}$ is −3.87 eV. The chemical hardness is a measure of resistance to charge transfer and its inverse gives softness. The negative value of chemical potential in TQ-DPPH$^{\bullet}$ is indicative of the stability of the complex. Lower stability would correspond to better hydrogen donation that needs better antioxidant property. The electrophilic index defines the affinity of electrons. The information obtained from global molecular descriptors confirms that the compound TQ tended to donate electrons to DPPH$^{\bullet}$ rather than attract electrons [68].

Table.4.6. Global molecular descriptors of TQ-DPPH$^{\bullet}$ in Ev

Global molecular descriptors	Values in eV
Ionization potential (IP)	-7.30
Electron affinity (EA)	-3.87
Chemicalhardness(η)	-5.36
Softness (S)	-2.68
Chemical potential(μ)	5.36
Electronegativity index (ω)	-9.23

4.4 Molecular docking

Molecular docking is an effective In silico method implemented to identify the protein-ligand binding site and obtain the binding affinity of the binding site. The structure of BSA is made up of three homologous a-helical domains I, II and III, each domain is constituted by two subdomains A and B. The ligand binds to BSA at two major binding site

namely sudlow site I and site II which are found at subdomain IIA and IIIA respectively [66]. The blind docking confirmed that the binding site is identified in subdomain III A at sudlow site II for BSA and TQ interaction. The molecular docking conformations of BSA-TQ with lowest binding energy are presented in the Fig. 4.7 respectively. The binding affinity of BSA-TQ has been found to be -5.7kcal/mol. During the interaction, the amino acids residues that surrounding TQ in the binding site I were Arg435, Lys431, Tyr451, Leu454.The binding of TQ with BSA protein occurs by hydrogen bond interaction through hydroxyl groups of TQ and polar residues of BSA, as well as hydrophobic interaction between TQ planar ring surfaces and non-polar residues of BSA.[69] The interaction plot produced by Ligplot is given in Fig.4.8

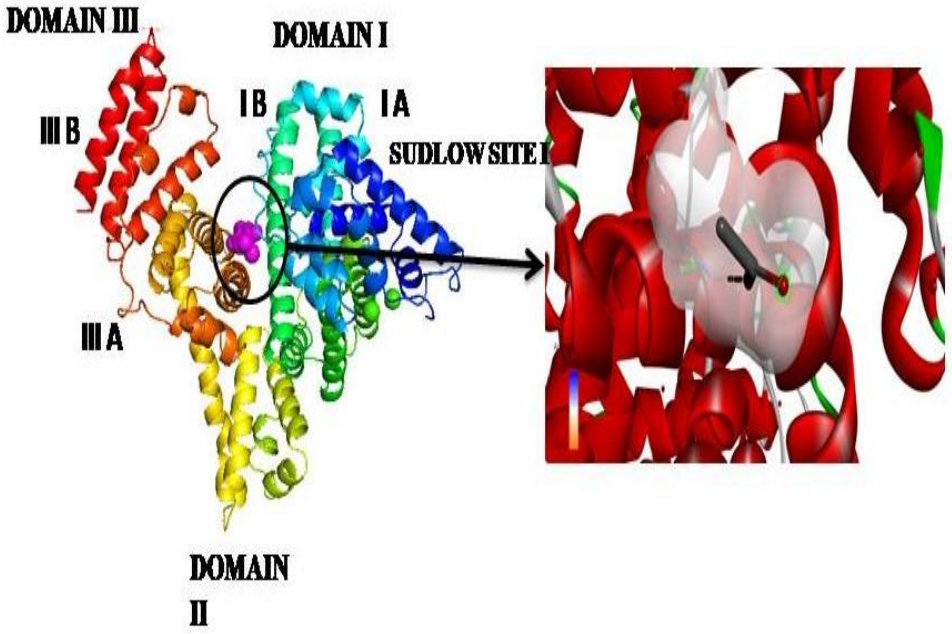

Fig4.7 The molecular docking confirmation of TQ-DPPH with high binding affinity

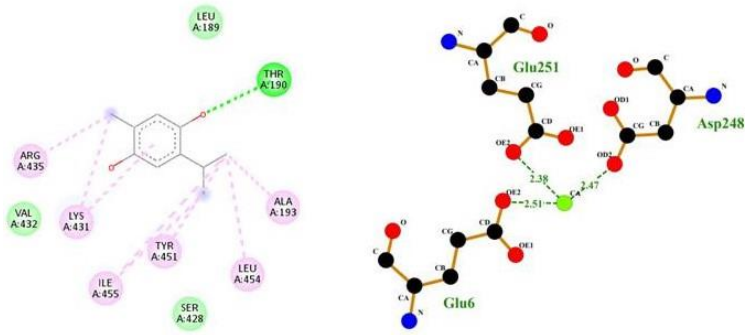

Fig4.8 Two dimensional representations of ligplot of hydrophobic interaction residues of BSA with TQ

Show the possible hydrophobic interaction between residues of BSA and TQ. From the Fig.4.8 BSA encircled by TQ at site I are Lys211, Val215, Asp323, Leu326, Gly327 and Leu330. The hydrophobic interacting residues labelled in red arcs surrounded by ECT at site II are Ala193, Arg196, Leu189, Ser428, Lys431, Leu454 and Arg458 shown in Fig. 5b. ECT bound to BSA showed considerably high number of hydrophobic contacts than CT. The structural stability of the biomolecular complexes is sustained by the formation of hydrogen bond. In the case of the BSA-CT complex, the three hydrogen bonds are formed between TQ and the amino acid residues Glu 251, Glu 6, and Asp 248 with bond distance of 2.38, 2.51 and 2.47 Å respectively (Fig. 8). Hence, the TQ exhibits more stable and strong complex formation with BSA protein than TQ by both hydrophobic and hydrogen bond interactions The above result supports the experimental findings investigated from fluorescence analysis. Further, the existence of hydrophobic and hydrogen bond interactions between Thymoquinone and the central cavity of milk protein bovine beta-actoglobulin are confirmed by Al-Shabib et al.

SUMMARY AND CONCLUSION

CHAPTER 5
SUMMARY AND CONCLUSION

The extraction from seeds of *Nigella sativa* L. (NSE) is the richest source of antioxidants which has been widely used to protect the cell membrane from oxidative damage. HPLC techniques are implemented to observe the retention time of NSE and the standard thymoquinone (TQ). The obtained retention time of the extract confirms the presence of the compound TQ in the NSE. The radical scavenging activity of TQ has been investigated using 2,2-diphenyl-1-picrylhydrazyl(DPPH$^{\bullet}$)assay. The experimental results suggest that TQ possess antioxidant potential through either through hydrogen atom transfer (HAT) or single electron transfer (SET) mechanism.The antibacterial activity showed that both NSE and TQ exhibited highest activity against *P.aeruginosa* than *S.aureus*. The formation of hydrogen bond interaction between TQ and DPPH$^{\bullet}$ has been examined using electronic structure calculation at B3LYP/6-311g ++ (2d, 2p) level of theory. It is observed that the bond length of C-H at quinone moiety, N-N and N-C at picryl ring are slightly elongated because of interactions of TQ with DPPH$^{\bullet}$. The non-covalent interactions that occur at TQ-DPPH$^{\bullet}$ complex is confirmed by quantum theory of atoms in molecules and topological analysis. Intermolecular charge transfer by hydrogen bonding interaction has been predicted using NBO method. The nature of electron transfer from TQ to DPPH$^{\bullet}$ are analyzed by the frontier molecular orbital analysis. Molecular docking analysis confirmed the binding of TQ with BSA protein occurs by hydrogen bond interaction through hydroxyl groups of TQ and polar residues of BSA, as well as hydrophobic interaction between TQ planar ring surfaces and non-polar residues of BSA.

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