Evaluation of Phytochemical Antioxidant Properties and Quantification of Thymoquinone in *Nigella sativa* L. Seed Extract

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Abstract: Herbal drugs have been considered to be a beneficial remedy for various diseases. They are considered to have fewer side effects compared to modern medicine. In the world of herbal medicine, *Nigella sativa* L. (Ranunculaceae), is one such herb held in high esteem owing to its notable pharmacological efficacies like neuroprotective and antioxidative properties. The present study aims to analyse the polyphenolic content, and antioxidant properties, and to quantify Thymoquinone (TQ) in *N. sativa* extract. Ethanol extract was screened for possible antioxidant activity against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Hydroxyl radical, Superoxide anion radical and Hydrogen peroxide. Further, quantification of TQ was performed by HPLC. The results confirmed that *N. sativa* extract possesses positive antioxidant properties, which can be attributed to the presence of bioactive compounds. Thus, this extract can be considered as an effective agent with high therapeutic value in preventing cellular damage. Exploration of these chemicals might lead to novel drug discovery which might be an effective remedy for neurological disorder.

Keywords: *Nigella sativa*, Antioxidant activity, Thymoquinone, HPLC, Ethanol extract

1. Introduction

Reactive oxygen species (ROS) have been one of the leading mechanisms that correlates with oxidative damages caused by generation of free radicals and leads to chronic diseases like diabetes, cancer, neurodegenerative disease and aging [1]. For many years, researchers have been looking for effective and non-toxic antioxidants from natural resources, especially medicinal plants for the prevention and treatment of ROS mediated diseases. A special emphasis has been laid on antioxidant therapy that can inhibit the oxidation of lipid and other molecules by inhibiting the initiation of oxidative chain reactions [2]. Thus, attention has been increasingly paid towards development and utilization of more effective and non-toxic antioxidants [3]. Nature has been a resource of medicinal treatments for thousands of years. There has been an increase in the use of products derived from plants and their purified bioactive compounds as alternative medicines which show favourable therapeutic advantages [4, 5]. *Nigella sativa* Linn (Black cumin or Black seeds) belonging to the family Ranunculaceae is an annual herbaceous plant commonly found in the Mediterranean countries, Middle East, Eastern Europe and Western Asia [6]. *N. sativa* seeds are a source of traditional herbs used in the Middle East as folk medicine and natural remedies due to its various biological properties [7]. Phenolic and flavonoid compounds are primarily responsible for the free radical scavenging and antioxidant activities of plants which play a beneficial role in medicine and have extensive pharmacological properties [8].

The seeds of *N. sativa* have been subjected to a wide range of pharmacological investigations due to the presence of phenolic and other active components such as carvacrol, thymol, thymoquinone, dithymoquinone and nigellin which exhibit a wide range of biological effects, including antioxidative, neuroprotective, anti-tumour, anti-fungal, analgesic, and anti-inflammatory [9,10]. Thymoquinone (2-methyl-5-isopropyl 1-1, 4-benzoquinone, also called TQ) is the major constituent of *N. sativa* seeds and is also the main oxidation product of carvacrol and thymo [11]. It is reported that these compounds have free radical scavenging properties and a good reducing power. The extraordinary biological activity of *N. sativa* is attributed to its oil component and thymoquinone [12, 13].

The remarkable advances in understanding the mechanisms of action of antioxidants against oxidative stress have brought their importance on human health to light. Natural antioxidants are also in high demand for application as nutraceutical or functional foods and biopharmaceuticals due to its consumer preferences and their therapeutic effect. Additionally, some human epidemiological studies have shown that natural antioxidant have potential health effects [14]. However, there is a dearth of scientific information on antioxidant potential of *N. sativa* seed to justify its continuous use in traditional folk medicine. Therefore, the present study aims to evaluate the quantity of Thymoquinone, polyphenolic contents and free radical scavenging potential of *Nigella sativa* L. ethanolic seed extract with a view to determine its potential health benefits.

2. Materials and Methods

Plant material

The *Nigella sativa* seeds were purchased from a local market in Bangalore, Karnataka. The seeds were dried at an ambient condition and were powdered. Powdered seeds were subjected to Soxhlet extraction using ethanol as a solvent. It was then filtered and dried by using rotary evaporator, and preserved at -4°C until further analysis.
Chemicals
Gallic acid, Trichloroacetic acid (TCA), ferric chloride, nicotinamide adenine dinucleotide (NADH), butylated toluene (BHT), nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), quercetin, ascorbic acid, thymoquinone were obtained from Hi media, Sigma Aldrich India Ltd

Phytochemical Screening

Estimation of Total phenolic (TP) content:
The amount of total phenolic content was determined according to the modified method of Prabhu et al., using by Folin- Ciocalteu reagent [15]. Gallic acid (0.5mg/mL) was used as the standard. 1 mLaliquot of various concentrations of gallic acid ranging from (1-125mg/ mL) and 5mL of FC reagent (1:10 diluted with distilled water) were mixed. After 5 min, 4mL of sodium carbonate solution was added and the solution was incubated for 30 min at a room temperature. The Absorbance of the samples was measured at 765nm using a UV Vis spectrophotometer. The contents were calculated in gallic acid equivalents (GAE) using the following formula.

\[ X = C \frac{V}{m} \]

Where

X= Phenol content, mg/g plant extract in Gallic equivalents; 
C= Gallic acid concentration from the calibration curve in mg/g 
V= Volume of plant extract, mL 
m= Weight of the plant extract, g

Estimation of Total flavonoid (TF) content:
The amount of total flavonoid content was estimated spectrophotometrically by following the modified protocol given by Bhatti et al., [16].This method is based on the formation of flavonoid aluminium complex. 0.5mL of the sample was mixed separately with 0.1mL of aluminium chloride solution (2% w/v). After incubation at room temperature for 15min, the absorbance of the reaction was measured at 510nm. The flavonoid content was calculated from the standard Quercetin curve and the results are expressed as quercetin equivalent (QE) using a similar formula used for total phenols.

Estimation of Total tannin content
The amount of tannin content was estimated by modified method of Stalin and Sudhakar [17]. The method is based on the mechanism by which phenols reduce potassium ferrous cyanide to produce ferrous ions; these ferrous ions in turn react with ferric chloride in the presence of dilute HCl to form a Prussian blue coloured complex, which can be measured spectrophotometrically at 700nm. The tannin content was calculated from the standard tannic acid curve and results the expressed as tannic acid equivalent (TAE) using a similar formula used for total phenols and flavonoids.

DDPH radical scavenging activity
The DPPH quenching ability of N.sativa ethanolic extract was measured by DPPH radical scavenging assay described by Rehmanet al., [18].This method depends on the reduction of purple 1,1-diphenyl-2-picrylhydrazyl (DPPH) to a yellow coloured diphenyl- picrylhydrazine and the remaining DDPH which showed maximum absorption at 517nm which was measured spectrophotometrically. 1mL of extract of varying concentrations (1-100μg/mL) and quercetin standard were mixed with 3mL of freshly prepared DDPH in methanol. An equal amount of methanol and DPPH were served as control. The solutions were mixed thoroughly and incubated in dark for 15 min at room temperature. The absorbance of sample and standard was measured at 517nm. The decrease in absorbance ofDDPH solution indicates an increase in DPPH radical scavenging activity. The ability to scavenge the DPPH was calculated using the following equation.

\[ \text{DPPH radical scavenging activity} = \frac{(A_o - A_1)}{A_o} \times 100 \]

Where:

Ao is the absorbance of the control (DDPH) and A1 - is the Absorbance of the sample.

Percentage radical activity was plotted against the concentration of the corresponding antioxidant substance to obtain the IC₅₀ value. IC₅₀ is defined as the amount of antioxidant substance required to scavenge 50% of free radicals present in the assay system. IC₅₀ values are inversely proportional to the antioxidant potentials. The analysis of the sample was done in triplicates.

Hydroxyl radical scavenging assay
The 2- deoxyribose assay was used to determine the effect of the extract on –OH radical [20]. Various concentrations of 0.4mL extract were mixed with 0.6mL of deoxy ribose (1mM) and the volume was made to 1.6mL using phosphate buffer. The reaction mixture was incubated for 10 min at an ambient temperature and 0.4mL of phenyl hydrazine hydrochloride (0.2mM) was added. The reaction mixture was incubated for 1hour after adding 1mL each of 2.8% TCA and 1% TBA. It was then heated on boiling water bath for 10 min and absorbance was measured at 532nm. The negative control without any antioxidant was considered 100% deoxyribose oxidation. The percentage of hydroxyl radical scavenging activity of extract was determined by comparing with the negative control. Ascorbic acid was used as the standard.

Hydrogen peroxide scavenging assay
Hydrogen peroxide radical scavenging activity of N.sativa extract was determined by adding 1mL of 1M hydrogen peroxide to 1mL of extract. 3% of ammonium molybdate was added followed by 2M sulphuric acid and 1.8mM
potassium iodide. The mixture was titrated against 5.09 mM sodium thiosulphate. The end point was yellow to colourless. The reaction mixture without extract was used as control. Ascorbic acid was used as the standard [21].

The percentage inhibition was calculated using the formula, 
\[
\% \text{ Inhibition} = \left( \frac{V_0-V_1}{V_0} \right) \times 100
\]
Where, 
- \( V_0 \) - Volume of sodium thiosulphate used by control
- \( V_1 \) - Volume of sodium thiosulphate used by extract.

Quantification of Thymoquinone (TQ) by HPLC:
Preparation of Thymoquinone standard for HPLC analysis. Thymoquinone (99.9% purity), standard was procured from Sigma Aldrich, Bengaluru. Standard solutions of TQ 0.2%, 0.4%, 0.6% and 0.8% w/v were prepared in methanol by dilution from stock solutions and were used for verification of retention time and preparation of calibration curve.

Chromatographic procedure
Thymoquinone was quantified by HPLC (High Pressure Liquid Chromatography) in a Shimadzu HPLC model. The elution was carried out in Luna 5 µm C18 (2)100 Å column with dimension of 250 × 4.6mm, 5µl. Acetonitrile: HPLC grade water (55:45, v/v) is used as mobile phase with flow rate of about 1mL/min. PDA (λ=254nm) is used for quantification of thymoquinone. The total run time was for 14 min. About 20 µL of the standard (Thymoquinone) and N. sativa extract and was injected separately and respective chromatograms were analysed [22].

Statistical Analysis:
All data were reported as mean ± standard deviation of means of three replicates. The IC₅₀ were calculated using Graph Pad Prism 6 Software.

3. Results

Total phenolic, flavonoid and tannin content
The total phenolic content of N. sativa extract was expressed in terms of gallic acid equivalent (GAE). The concentration of total phenol is expressed as mg of GAE/g extract, the total phenolic content in the extract was found to be 8.248 ±0.33 mg GAE/g.

The total flavonoid content of N. sativa extract is expressed in terms of quercetin equivalent (QE). The concentration of total flavonoid is expressed as mg of QE/g of extract. The total flavonoid content in the extract was 4.62±0.01 mg QE/g.

The total tannin content of N. sativa extract is expressed in terms of tannic acid equivalent (TAE). The concentration of total tannin is expressed as mg of TAE/g of extract. The total tannin content in the extract was 0.59±0.01 mg TAE/g.

DPPH radical scavenging assay
The antioxidant potential of the ethanolic extract of N. sativa was analysed by DPPH radical scavenging assay. Fig 1 suggests that the extract exhibits a dose dependent activity, i.e. DPPH scavenging activity increases proportionally with increase in concentration of the extract. The IC₅₀ value of the extract was found to be 46.01 µg/mL and that of standard quercetin was 2.02 µg/mL. The ethanolic extract of N. sativa showed moderately significant scavenging effects on DPPH radical.

Superoxide radical scavenging assay:
The percentage inhibition of super oxide radical generation of N. sativa ethanolic extract was found to be increasing in a concentration dependent manner as illustrated in fig 2. The IC₅₀ value and the standard ascorbic acid value was found to be 46.26 µg/mL, 80.46 µg/mL respectively

Hydroxyl and Hydrogen peroxide radical scavenging assay:
This assay shows the ability of N. sativa extract and standard ascorbic acid to inhibit hydroxyl radical mediated deoxyribose degradation. The results indicate that the extract inhibition occurs in a dose dependent manner as per Fig 3. The IC₅₀ value of the extract at 65.24 µg/mL was less than that of standard Ascorbic acid at 56.21 µg/mL. The result obtained from the hydroxide scavenging assay shows that the inhibition of N. sativa at 53.53% was less than that of standard Ascorbic acid at 46.1%.
Quantification of Thymoquinone (TQ) by HPLC

The ethanolic extract of *N. sativa* was subjected to HPLC analysis to quantify the amount of TQ present. This analysis demonstrated the presence of TQ, the bioactive compound. Its quantity was found to be 0.32%. The linear regression equation was obtained in the form of \( Y = mx + c \), where \( y \) and \( x \) corresponds to peak area and concentration, respectively (Fig 4a). A comparison of HPLC profiles of the ethanolic extract with that of thymoquinone showed a retention time of 10.53min (Fig4b and Fig4c).

![Figure 4 (a): Calibration curve for standard Thymoquinone](image)

![Figure 4 (b): HPLC peak for standard Thymoquinone.](image)

![Figure 4 (c): HPLC peak for *N. sativa* L. seed extract](image)

4. Discussion

Recently appropriate assessments of antioxidant activity as well as availability of resources in the form of food and medicinal plants are drawing great attention in food science and nutrition. Plants are a good source of antioxidants, the presence of polyphenols such as phenols, flavonoid and tannins play a major role in scavenging free radicals [23,24].Free radicals are responsible for unregulated chain of oxidation and reduction reactions. Oxidative stress in the form of reactive oxygen species (ROS) is generated due to various environmental factors such as pollutants, pathogen invasion, toxins and ultra violet radiation. The presence of ROS is extremely damaging to the organism at high concentration. The increased production of ROS due to environmental stress leads to various disease conditions such as cancer, neurodegerative diseases, diabetes and other inflammatory diseases [25]. Many synthetic drugs protects against oxidative damage but they have adverse side effects, substitute to this problem is to consume natural antioxidants.

Plants metabolite includes aromatic compounds such as phenols. Phenols exhibit anti-aging, anti-inflammatory and anti-apoptotic properties. Flavonoids are potent antioxidants. The potency depends on the molecular structure and the position of hydroxyl group within the chemical structure. Tannins are medicinally used for their antiviral, antibacterial and anti-tumour activities. Certain kind of tannins can reduce the mutagenicity of a number of mutagens and also exhibit antioxidant activity [26,27]. It is evident from the study that *N. sativa* extract shows a higher phenolic, flavonoid and tannin content (8.248 ±0.33 mg, 4.62±0.01mg and 0.59±0.01 mg) and is a good source of polyphenols. Toma et al., [28] reported the presence of total phenols 4.12mg and flavonoid 2.01mg content in the crude ethanolic extract of *N. sativa*. Similarly, Goga et al., [29] reported 5.58mg of phenolic content and 3.99mg of flavonoid content in crude ethanol and 31.5mg of phenolic and 16.34 mg flavonoid content in n-hexane extract of *N. sativa* respectively. Hence, the present study is in line with observations from earlier studies. Further, the role of these secondary metabolites in the maintenance of anti-oxidative approach is well elucidated.

The antioxidant activity of *N. sativa* extract was determined using DPPH, a stable radical commonly used to determine scavenging activity. In the present study, it was found that the scavenging activity increased with increase in concentration. The results from the studies on anti-oxidant property of *N. sativa* extract demonstrated a significant scavenging activity. The IC\(_{50}\) value was found to be 46.01 μg/μL when compared to the standard quercetin 2.02 μg/ μL. Similar studies in petroleum ether, distilled water and methanol revealed a dose dependent increase in scavenging activity [30]. Toma et al. highlight the scavenging ability of *N. sativa* crude ethanolic extract that showed IC\(_{50}\) = 624.7 ± 12.77 μg/ μL [28]. The result of the present study confirms that the ethanolic extract of *N. sativa* contains sufficient phytochemical constituents with the ability to donate ‘H’ to DPPH free radical and reduce it to a non-free radical form viz., DPPH-H.

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Superoxide anion radical (O$_2^-$) is generated by four electron reduction of molecular oxygen in to water. This radical also forms in aerobic cells due to electron leakage from the electron transport chain. O$_2$ radicals are very harmful to cellular components and increase in number under stress conditions [31]. Inhibition of superoxide anion radical derived from dissolved oxygen by FMS/NADH coupling reaction reduces NBT in PMS/NADH-NBT system. It is reported that flavonoids are effective antioxidants mainly because they scavengesuperoxide anions. In this study an assessment was made to confirm invitro super anion scavenging activity in N.sativa seed extract. The IC$_{50}$ value was found to be 80.46 μg/mL when compared to standard ascorbic acid (46.26 μg/mL). Rather et al showed moderate to strong free radical scavenging activity of N. sativa oil [32]. Kamili [33] reported that petroleum ether extract of N. sativa exhibits good superoxide anion radical scavenging activity (85%). The present study has shown that superoxide radical activity of N. sativa and standard ascorbic acid are markedly increased with increase in concentration and suggests that the extract possesses notable quenching activity due to the presence of phenolic compounds.

Hydrogen peroxide (H$_2$O$_2$) is a weak oxidising agent that directly inactivates some enzymes, usually by the oxidation of essential thiols (-SH) groups. H$_2$O$_2$ plays a major role in signalling and regulates a wide variety of biological functions. It can cross the cell membranes rapidly, and can react with Fe$^{2+}$ and Cu$^{2+}$ ions to form hydroxyl radicals which is the origin of many toxic effects [34]. An earlier study by Nejdet et al, reported that the reducing power of N.sativa in methanolic extracts increased with increasing concentration [35]. The present study, demonstrate that the ethanolic extract shows of N. sativa exerted significant H$_2$O$_2$ scavenging activity on peroxide radicals. N. sativa showed activities of 53.5% and 46.1%, respectively.

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and oxidative damage of DNA. The interaction of ferrous or cupric metal ions with hydrogen peroxide leads to the formation of hydroxyl radical in biological systems [36,37]. The hydroxyl radical generated by the Fe$^{2+}$ ascorbic acid and EDTA –H$_2$O$_2$ system was scavenged by the extract and the standard in a concentration dependent manner. This observation suggests that N. sativa can be used as an alternative remedy to synthetic antioxidants in combating the oxidative activity of hydroxyl radical.

The HPLC analysis in the present study revealed a 0.32% composition of thymoquinone in the ethanolic extract of N.sativa. According to Jumah et al, the lowest percentage composition of thymoquinone was observed in the aqueous extract. The solubility and stability of thymoquinone were studied and it was suggested that the content of thymoquinone may differ as a result of various factors of extraction [22]. A lesser content of thymoquinone in ethanol and methanol extracts was reported [38,39]. As per Ashraf et al., the antioxidant potential of thymoquinone varies depending upon the choice of solvent or extraction procedure [40].

5. Conclusion

Phytochemical investigation of ethanolic extract of N. sativa revealed the presence of polyphenolic compounds and favourable free radical scavenging activity. N. sativa extract showed the presence of Thymoquinone, which is the main bioactive compound, used traditionally in the treatment of various diseases. Hence, this plant alone or in combination may exhibit a distinguished antioxidant activity and help to curb free radicals such as ROS and is recommended a substitute for synthetic drugs to handle oxidative damage caused to various biological macromolecules, including DNA, lipids and proteins. Further isolation of bioactive compounds and evaluation of therapeutic values in living models is required for novel drug discovery.

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