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Peroxidase-Mediated Thymoquinone Biosynthesis in *Nigella sativa*: A pH-Dependent Enzymatic Insight

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Abstract

Nigella sativa (N. sativa) is widely recognized for its pharmacological potential, largely attributed to thymoquinone (TQ), a key bioactive compound. Although TQ biosynthesis has been reported in various plants and microorganisms, its enzymatic pathway in N. sativa remains inadequately characterized. This study investigates the role of peroxidase enzymes in TQ biosynthesis, using thymol-a known precursoras the substrate, hypothesizing that the antioxidant capacity of N. sativa arises from robust enzymatic defense mechanisms. Peroxidase activity was evaluated in the leaves, buds, and fruits of the Rajendra Shyama cultivar using 1% hydrogen peroxide (H_2O_2) in Tris buffer at different pH 6.5, 7.0 and 7.5. Absorbance analyses at 254 nm and 274 nm were used to monitor TQ and thymol formation, respectively. Results revealed plant part-specific variations in enzymatic activity, with leaves unveiling the highest and fruits the lowest peroxidase activity. Optimal activity was observed at pH 6.5, which suggests a preference for slightly acidic to neutral conditions for effective TQ biosynthesis. Notably, differential absorbance patterns and correlation studies indicate the possible involvement of metabolites other than thymol in the biosynthetic pathway. These findings provide new insights into the enzymatic dynamics of N. sativa, with significant implications for therapeutic exploitation and metabolic engineering.

Keywords: Antioxidants, *Nigella sativa*, Peroxidase, Thymol, Thymoquinone.

Introduction

Nigella sativa (*N. sativa*) Linn., a dicotyledonous annual herb native to the Mediterranean region, is globally recognized for its traditional medicinal uses[1]. The plant grows up to 45 cm tall, having blue flowers and produces small, black, funnel-shaped seeds[2]. Nutritionally, the seeds are rich in vegetable proteins, fiber, minerals and vitamins. Phytochemical analyses have revealed hundreds of compounds, especially alkaloids, saponins, sterols and essential oils[3]. Major constituents include saturated and unsaturated fatty acids, nigellone, thymoquinone (TQ), thymohydroquinone (THQ), dithymoquinone (DTQ), thymol (Thy), carvacrol (CV), α-pinene, d-limonene, p-cymene and α-hederin[4,5].

TQ, a monoterpene quinone (2-isopropyl-5-methylbenzo-1,4-quinone), is the principal bioactive component responsible for most pharmacological effects of *N. sativa*[6]. These include antimicrobial, antiviral, immunomodulatory, anti-inflammatory, antioxidant, anticancer, neuroprotective and gastroprotective activities[7]. Furthermore, TQ shows therapeutic potential in oral health, cardiovascular and reproductive disorders, respiratory ailments and bone-related conditions[8,9].

Known for its therapeutic importance, the biosynthetic pathway of TQ has encouraged extensive research. Key intermediates in TQ biosynthesis include γ-terpinene, p-cymene, CV and Thy[10]. Enzymes such as monoterpene synthase, geraniol synthase, geranyl diphosphate synthase, superoxide dismutase, alcohol dehydrogenase and peroxidases are implicated in this pathway. These metabolites and enzymes not only found in *N. sativa*, but are also found in plants like thyme, oregano and peppermint[11,12], as well as microbes such as *Streptomyces*[13] and *Castellaniella defragrans*[14].

Peroxidases are oxidoreductase enzymes that catalyze the oxidation or reduction of varied substrates. In the presence of hydrogen peroxide (H_2O_2), they are crucial for biotransforming phenols, aromatic amines and other organic compounds[15]. Experimental studies have shown that peroxidases efficiently oxidize phenolic compounds into their corresponding quinones, which generates bioactive molecules involved in plant defense and secondary metabolism[16]. Class III plantperoxidases (secretory) participate in removal of H_2O_2, lignin biosynthesis and suberization[17]. In plants, these functions lead to the quinone formation, such as TQ in *N. sativa*. Notably, peroxidases also have importance for human health, including pathogen killing via myeloperoxidase and protection against oxidative damage via glutathione peroxidases[18,19].

Recent studies have further highlighted the dual importance of these enzymes. Rasuli et al.[20] have demonstrated the enhanced Thy and CV biosynthesis in *Thymus vulgaris* via biostimulant treatment, which underscores the peroxidase-mediated regulation of monoterpene-derived quinones. Simultaneously,

Jiang et al.[21] have reported the novel therapeutic applications of plant-derived peroxidases in oxidative stress management.

Taking into consideration, the role of peroxidase and Thy in TQ biosynthesis, this study was undertaken to investigate the enzymatic involvement of peroxidase using Thy as a substrate. Our previous HPLC analysis suggested Thy and CV as the key intermediates. This work aims to identify the most likely substrate and clarify the enzymatic steps leading to TQ formation. By addressing this mechanistic gap, the study offers insights into the bioconversion of the cost-effective molecule Thy into high-value TQ.

Materials and Methods

Plant Material: The Rajendra Shyama (RS) cultivar of *N. sativa* was selected as the standard for investigating enzymes involved in the conversion of Thy to TQ. Leaf, bud and fruits were collected to assess hydrogen peroxide (H_2O_2)-dependent oxidoreductase activity, with a special focus on peroxidase enzymes[10,22,23].

Homogenate Preparation: Fresh plant parts (0.25 g) were washed with double-distilled water and blot-dried. A 20% (w/v) homogenate was prepared using extraction buffer containing 100mM Tris-HCl (pH 7.0) and 0.1% insoluble polyvinyl polypyrrolidone (PVPP) to remove phenolic compounds and prevent enzyme inhibition[24]. The homogenate was incubated on ice for 15 minutes and centrifuged at 4696.8 × g at 4°C for 15 minutes. The clear supernatant was collected in another centrifuge tube and used as the crude enzyme extract.

Protein Estimation: The protein content of enzyme extracts was quantified following the standard Bradford assay[27], using bovine serum albumin/ BSA (Sigma, USA) as the calibration standard.

Reaction Mixture and Enzymatic Assay: Enzyme activity assays were performed for leaf, bud and fruits at pH 6.5, 7.0 and 7.5. The reaction mixture contained 100 mM Tris buffer, 0.3% H_2O_2 as the electron acceptor and 1µg/mL Thy as the substrate. Enzyme activity was monitored spectrophotometrically at 254 nm and 274 nm to detect changes in absorbance corresponding to TQ and Thy, respectively[28-30]. Activity values were expressed as relative absorbance units.

Statistical Analysis: All the data were analyzed independently and subjected to correlation analysis to evaluate relationships among the measured variables. Activity values were expressed as relative absorbance units, providing a measure of substrate oxidation under varying conditions.

Results and Discussion

The peroxidase activity was evaluated at 254 and 274 nm to find its involvement in Thy to TQ conversion, if any. The three pH levels were selected to monitor TQ formation in the leaf, bud and fruits of RS cultivar. When the activity profile was observed at 254 nm, it revealed clear pH-dependent variations (Figure-1). At pH 6.5, leaf showed the highest activity (0.478), followed by bud (0.344), while fruit exhibited the negative activity (-0.133). At pH 7.0 and 7.5, activity was declined across all the plant parts, with negative values recorded, suggesting optimal biosynthetic conditions are slightly acidic.When the spectrophotometric analysis was done at 274 nm, it revealed pH-dependent variations in thymol formation/consumption (Figure-2). In leaves, absorbance decreased progressively from -0.041 at pH 6.5 to -0.226 at pH 7.5. Buds showed a distinct pattern with partial recovery at pH 7.0, while fruits exhibited the most distinct decline in absorbance under alkaline conditions. These results demonstrate that thymol stability and conversion are most favorable between pH 6.5 and 7.0.

Further, correlation study was performed to monitor the relationship between TQ formation and Thy consumption. The result of correlation analysis is given in Table-1. In leaves, it showed the mechanistic evidence for efficient conversion by peroxidase from Thy to TQ. At pH 6.5 vs 7.0, a strong positive correlation at 254 nm (TQ formation) was accompanied by a strong negative correlation at 274 nm (Thy consumption), indicating efficient enzymatic conversion under near-neutral conditions. This aligns with the optimal activity range of class III peroxidases[15-17].

Similar observations were reported by Botnick et al.[22], who found increased TQ levels accompanied by decreased carvacrol/thymol in mature seeds. A strong negative correlation was observed at 254nm; while an average negative correlation was observed at 274 nm at pH 6.5 vs 7.5. The correlation analysis at pH 7 vs 7.5 revealed a negative correlation at 254 nm, while a positive correlation at 274 nm.

Table-1: Correlation between TQ and Thy absorbance in leaf, bud and fruit after enzyme activity assay at 254 and 274 nm, respectively.

Parameter	pH 6.5 vs 7	pH 6.5 vs 7.5	pH 7 vs 7.5
TQ 254nm	0.93	-0.93	-0.74
Thy 274nm	-0.93	-0.60	0.84

The correlation analysis at pH 6.5 compared to 7.5 indicated the inverse relationship between TQ formation and Thy disruption. It revealed a disruption in enzymatic activity, suggesting altered protonation states or conformational instability under mildly acidic conditions[31]. In contrast, persistence of Thymol was evident between pH 7.0 and 7.5, which indicated stabilization of its functional interactions within the near-neutral range[32,33]. The inverse relationship suggests peroxidase-mediated oxidation of Thy to THQ, followed by conversion to TQ. Similar variations

have been reported in other medicinal plants[23,25,26]. Our previous HPLC analysis revealed a variable relationship between Thy and TQ, directing to a complex regulation where environmental conditions alter the balance between precursor and product[34]. The results at pH 7.5 suggest that alkaline conditions disrupt the conversion. The higher activity in leaves is consistent with leaves being primary sites of secondary metabolite biosynthesis[35].

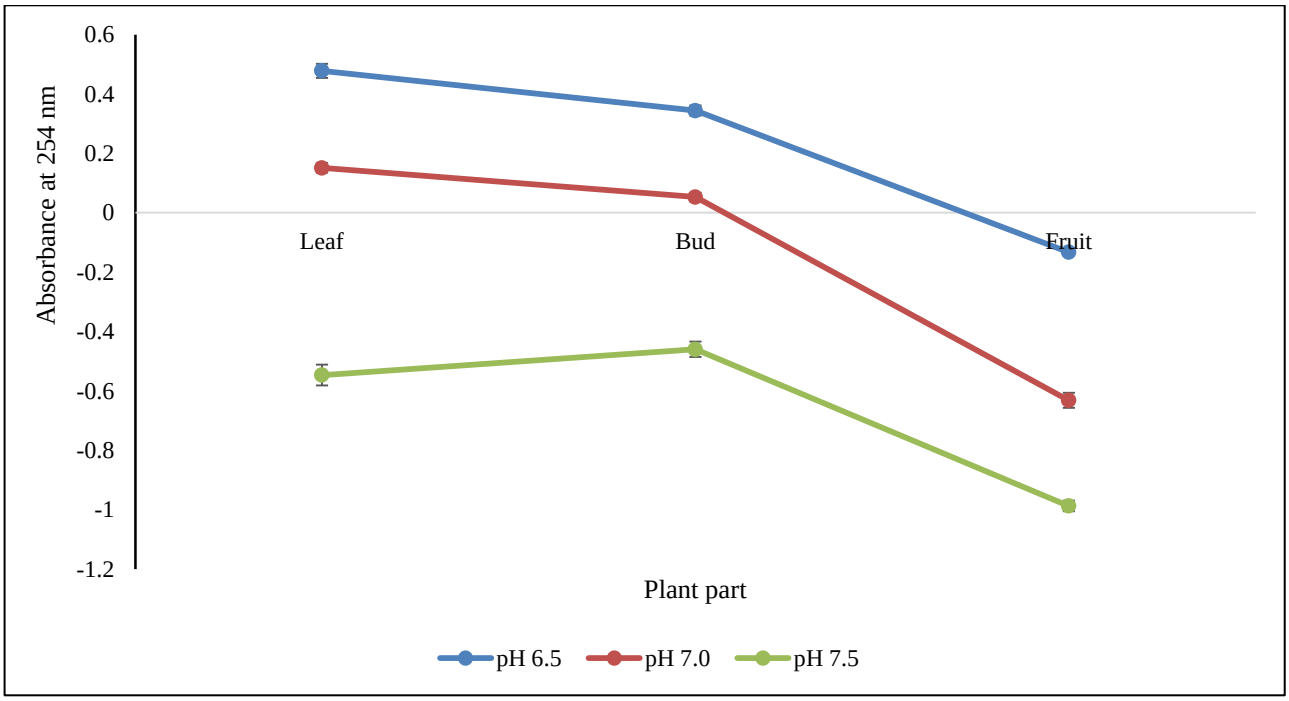

Figure-1: Change in absorbance at 254 nm for Thymoquinone formation in different plant parts at different pH.

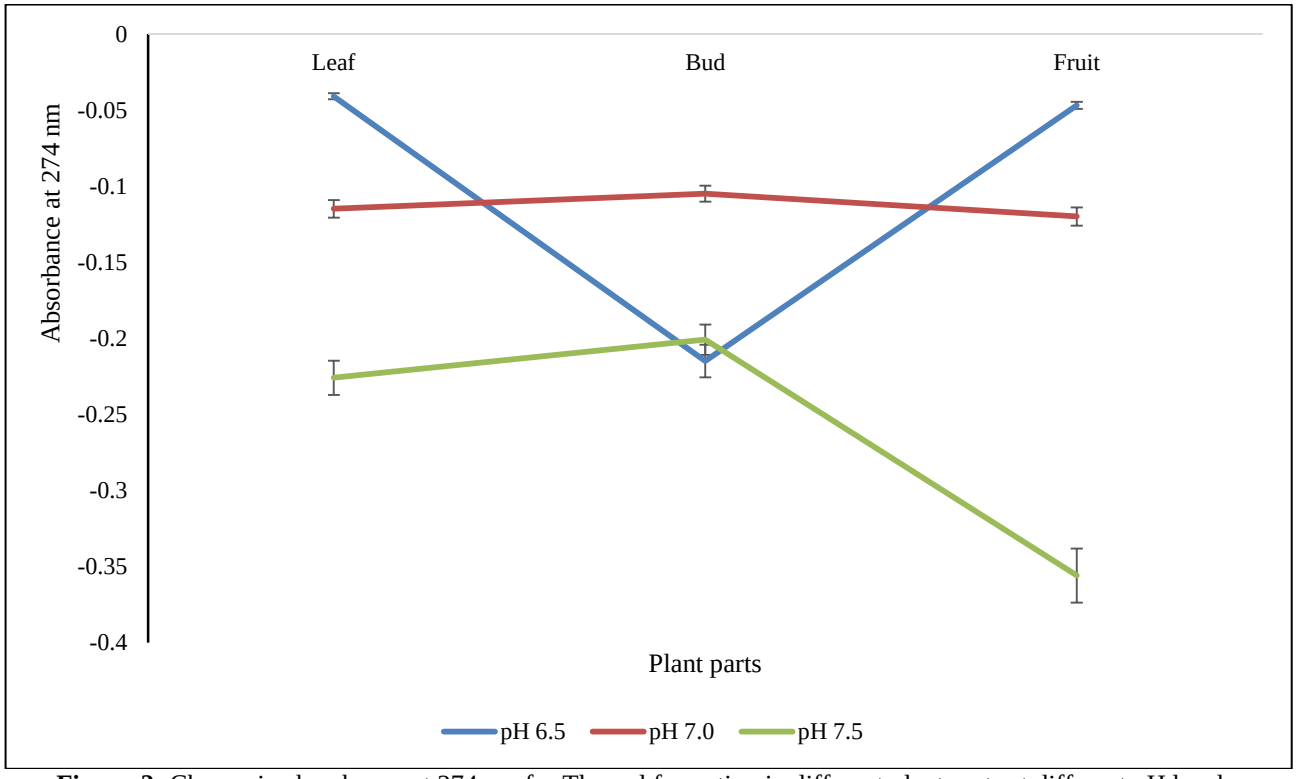

Figure-2: Change in absorbance at 274 nm for Thymol formation in different plant parts at different pH levels.

From a biosynthetic perspective, TQ formation begins with γ-terpinene and p-cymene, leading to Thy or CV. The current results support the hypothesis that peroxidase catalyzes the oxidative conversion of Thy to THQ, positioning Thy as the primary substrate[25,26,28]. Recent studies by Sadeghi et al.[29] and Shaukat et al.[30] reinforce this interpretation highlighting enzymatic regulation in TQ pharmacological pathways. Moreover, Alamri et al.[18] demonstrated the bioactivity of thymol derivatives as substrates in oxidative pathways. Several other studies have shown that engineered microbial systems can convert thymol to TQ using peroxidases[36,37], and electrochemical catalysis on Pt/CeO_2 catalysts can directly oxidize Thy to TQ[38]. Genome-wide studies further underline the role of peroxidase families in phenolic metabolism[39,40]. Overall, this study provides novel evidence for the pH-dependent activity of peroxidase in *N. sativa*, highlighting leaf at pH 6.5 as the most favorable condition for the enzymatic conversion of Thy to TQ.

Conclusion

In conclusion, this study demonstrates that peroxidase activity in the RS cultivar plays a pH-dependent role in thymoquinone biosynthesis. Thymol was identified as the most probable substrate for enzymatic conversion to thymohydroquinone and thento thymoquinone, while carvacrol appeared less likely to participate. The conversion was most constant between pH 6.5 and 7.0, whereas activity declined under alkaline conditions. This reflects the reduced enzyme stability and altered substrate dynamics. By clarifying this mechanistic step, this work advances the understanding of secondary metabolite regulation and highlights the potential of peroxidase-driven bioconversion of cost-effective thymol into high-value thymoquinone. These insights provide a foundation for biotechnological innovations, including enzyme-based conversion systems and metabolic engineering strategies, to enhance TQ yield in medicinal plants.

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