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## ANTIOXIDANT POTENTIAL OF *Citrus aurantium* ESSENTIAL OIL IN MITIGATING FISH OIL OXIDATION

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### Abstract

This study evaluated the antioxidant potential of *Citrus aurantium* flowers essential oil (neroli oil), in protecting fish oil, a lipid source highly susceptible to oxidation. Fish oil samples were supplemented with neroli oil (NEO) at concentrations ranging from 10 to 1500 ppm and subjected to rapid oxidation conditions (50°C for 48 hours with 70% humidity). Peroxide values (PV) were measured to assess oxidative stability, and significant differences were observed across treatment groups ( $p < 0.05$ ). The lowest PV ( $7.65 \pm 0.28$  meq O<sub>2</sub>/kg) was recorded in the 1500 ppm NEO group, while the control group without supplementation had the highest PV ( $29.54 \pm 1.02$  meq O<sub>2</sub>/kg). NEO demonstrated comparable effectiveness to the commercial antioxidant Butylated Hydroxytoluene (BHT, 200 mg/kg). Gas chromatography-mass spectrometry (GC-MS) analysis identified linalool (25.2%) and linalyl acetate (42.77%) as the major volatile components of NEO. The study highlights that NEO effectively suppresses lipid oxidation without exhibiting toxicity, making it a viable natural alternative for fish feed applications. Further investigations are recommended to explore the potential pro-oxidant effects at higher concentrations and the long-term implications of secondary oxidation products.

**Keywords:** Neroli oil, fish oil oxidation, natural antioxidants, *Citrus aurantium*, peroxide value

### Introduction

*Citrus aurantium*, also known as bitter orange, is a common plant belonging to the Rutaceae family. Its leaves, fruit, bark, flower and root are traditionally used to treat a wide range of diseases (Periyamayagam et al., 2013). NEO extracted from the flowers of the *Citrus aurantium*

tree has attracted great interest in recent years due to its various therapeutic properties and scientific research has begun to reveal the bioactive components associated with NEO and their potential benefits (Haj Ammar et al., 2012; Hsouna et al., 2013; Khodabakhsh et al., 2015; Scandurra et al., 2022). One of the main areas of interest in NEO is its potential as an antioxidant agent (Değirmenci & Erkurt 2020). Oxidative stress, which results from an imbalance between reactive oxygen species (ROS) production and the cellular antioxidant defense system, plays a role in the pathogenesis of numerous diseases, including cardiovascular disorders, neurodegenerative conditions and certain types of cancer (Aitken & Fisher 1994). NEO is known for its rich composition of bioactive compounds, including linalool, linalyl acetate, limonene, geraniol and various flavonoids. These components exhibit antioxidant properties and show the ability to scavenge free radicals, inhibit lipid peroxidation and modulate enzymatic antioxidant defenses. Furthermore, NEO has anti-inflammatory, antimicrobial and neuroprotective activities, which further contribute to its therapeutic potential (Değirmenci & Erkurt, 2020; Borba et al., 2021). Therefore, the identification of bioactive components of NEO and its potential for use as an antioxidant offers a promising approach in the prevention of oxidative stress. In this context, its effect on the protection of fish oil, which is particularly vulnerable to oxidation and has high nutritional value, against oxidation is the focus of this study. In addition, NEO was added as a potential feed additive in fish feeds and it was observed that it did not cause toxic effects in fish and even improved some physiological processes, especially growth, depending on the dose of use (Acar et al., 2021). Fish oil contains polyunsaturated fatty acids (PUFAs) that the human body and fish cannot synthesize and must take from outside through nutrition. Consumption of these fatty acids is known to have many health benefits, especially cardiovascular health, brain development and function, and eye health (Harris, 2004; Das et al., 2009). Addition to this fish oil is a critical source of nutrients for fish health and growth, with high levels of polyunsaturated fatty acids (PUFAs) found in fish feed. In particular, omega-3 fatty acids (EPA and DHA) play important roles in strengthening the immune system, improving growth performance and disease resistance of fish. However, PUFAs are highly susceptible to oxidation, which can lead to reduced nutritional value and poor feed quality. Preventing oxidation in fish feeds prolongs the shelf life of feeds and promotes healthy growth of fish (Tocher, 2010; Turchini et al., 2009). The presence of double bonds in polyunsaturated fatty acids renders unsaturated lipids prone to oxidation. Oxidation is a multifaceted reaction that often initiates with the generation of free radicals (Frankel, 1984; Min & Boff, 2002). Free radicals interact with oxygen to produce hydroperoxides. Hydroperoxides subsequently result in the synthesis of substances like aldehydes, ketones, and short-chain carboxylic acids, which are prone to decomposing into undesirable small molecules (Kazuo, 2019; Lembke & Schubert, 2014). Assessing the oxidation level of fish oils necessitates the measurement of lipid peroxide species and secondary oxidation products by methods such as peroxide value (PV) (Packer, 1999; Arneson & Roberts, 2007). Dietary consumption of lipid peroxides and secondary oxidation products stimulates the oxidation of other fatty acids in living organisms, resulting to the creation of more lipid peroxides in a chain reaction. This is believed to result in membrane peroxidation, cellular damage, and oxidative stress, which are recognized as pathogenic mechanisms in living organisms (Shahidi & Zhong, 2010). Consequently, unoxidized lipids ought to be ingested as dietary supplements, whereas the oxidation of fats should be inhibited by antioxidants. The incorporation of antioxidants is crucial for the oxidative stability of the oil, its shelf life, and an acceptable taste and odor for consumers. Synthetic antioxidants can safeguard fish oil against oxidation; nevertheless, they are not as safe as natural antioxidants.

The main goal of this study was to examine the efficacy of fish oil infused with varying quantities of NEO as an antioxidant against accelerated oxidation induced by temperature,

humidity, and continuous light exposure. Furthermore, the study compared its efficacy against widely used antioxidants such as Butyl Hydroxy Toluene (BHT).

## Material and Method

### *Volatile Components of Neroli Oil by GC-MS*

The volatile component profile of NEO was established through qualitative analysis using gas chromatography-mass spectrometry (GC-MS) (Shimadzu GCMS QP 2010 ULTRA). In the analysis, an RTX-5MS brand capillary column (30 m; 0.25 mm; 0.25  $\mu$ m) was employed, with helium serving as the carrier gas. The column oven temperature was established at 40°C, the interface temperature at 250°C, the ion source temperature at 200°C, and the injection temperature at 250°C. Injection volume was 1  $\mu$ L and split (1/5) technique was used for injection. The oven program was executed for 3 minutes at 40°C, followed by a temperature increase from 40°C to 240°C at a rate of 4°C per minute, maintained at 240°C for 10 minutes, then increased from 240°C to 260°C at 4°C per minute, and finally held at 260°C for 10 minutes, totaling 78 minutes. The peaks identified by the study were compared with the W9N11 library, allowing for the determination of the oil's volatile component profile (Kesbiç, 2019).

### *Antioxidant Activity of Neroli Oil*

The overall antioxidant activity was assessed by evaluating the scavenging impact of NEO and the commercial antioxidant BHT on the DPPH radical (DPPH: 2,2-difenil-1-pikrilhidrazil). To assess the scavenging effect of the antioxidants employed in the study on DPPH radical, a combination of 0.2 mL sample, 0.5 mL DPPH solution, and 4 mL 80% ethanol was vortexed for 15 seconds and subsequently incubated at room temperature in the dark for 15 minutes. At the conclusion of the period, the combination was analyzed using a UV-VIS spectrophotometer at 517 nm against the blank sample, and the absorbance was documented. The percentage of radical scavenging effect was determined using the formula presented in Sanna et al. (2012).

### *Rapid Oxidation Experiments*

Fish oil was acquired from Kobyalan Group, a company in Trabzon, Turkey, specializing in the production of fish oil for aquatic feed. Various amounts of NEO (10ppm, 100ppm, 500ppm, 1000ppm, 1500ppm) were solubilized in fish oil, and distinct experimental groups were formed to examine its protective impact against the thermal oxidation of fish oil. Butylated hydroxytoluene (BHT200) was utilized as a positive control at a dosage of 200 ppm. The experimental groups were subjected to treatment for 48 hours in temperature-resistant containers under ambient conditions of 50 $\pm$ 0.5 °C and 70% humidity (Kesbiç et al., 2023).

### *Peroxide Value*

The peroxide value determination method (Cd 8b-90) of the American Oil Chemists' Society (AOCS) was employed to assess the protective efficacy of NEO against oxidation under accelerated settings. Unoxidized fish oil and 0.5 g samples from oxidized experimental groups were dissolved in 5 mL of chloroform. The dissolved samples were incubated with 15 mL of acetic acid and 1 mL of saturated potassium iodide for 10 minutes at room temperature in darkness. Following incubation, titration was performed using 0.01 N sodium thiosulfate and few drops of 1% starch in 75 mL of deionized water as the indicator. The peroxide value was calculated using the formula based on the color shift that indicated the result of the titration.

$$PV (\%) = [(V1 - V0) N] / V$$

V1 and V0 represent the titrant volumes utilized for the sample and blank, respectively; N denotes the normality of the titration solution, and M signifies the sample weight. The peroxide value assay results were evaluated in triplicate as  $\text{meqO}_2 \text{ kg}^{-1}$  oil.

### Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were conducted with IBM SPSS Statistics 20 software. One-way ANOVA test was done to assess whether the differences between the sample groups were statistically significant, and then group comparisons were made with Tukey's HSD (Honest Significant Difference) test. In all analyses, a significance criterion of  $p < 0.05$  was established.

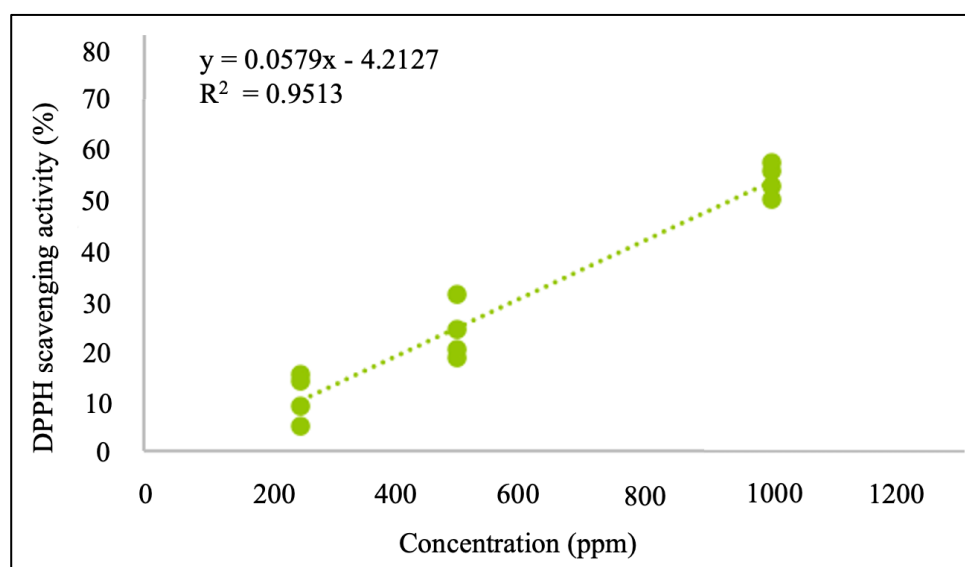
## Results

The volatile component profile of NEO is showed in Table 1. The research determined 89.28% of the volatile component concentration in NEO.

**Table 1.** Volatile component profile of NEO

Sn	Component	Retention Time (min)	Concentration (%)
1	Linalool oxide	15.166	2.21
2	Linalool	15.883	25.20
3	Acetic acid, 3-methyl-6-oxo-hex-2-enyl ester	18.137	0.42
4	$\alpha$ -Terpineol	19.248	3.26
5	Linalyl acetate	21.725	42.77
6	Geranial	22.137	0.64
7	3,7-Dimethyl-1,5-octadiene-3,7-diol	22.628	1.15
8	3-Nonanol, 1,2:6,7-diepoxy-3,7-dimethyl-, acetate	25.078	3.21
9	Geranyl acetate	26.069	6.92
10	D-limonene	29.949	3.50
<b>Total</b>			89.28

The IC<sub>50</sub> value of NEO was calculated using the curves presented in Figure 1. The IC<sub>50</sub> value of NEO in the present study was found as 936.31 ppm.



**Figure 1.** % DPPH scavenging activity of NEO at different concentrations.

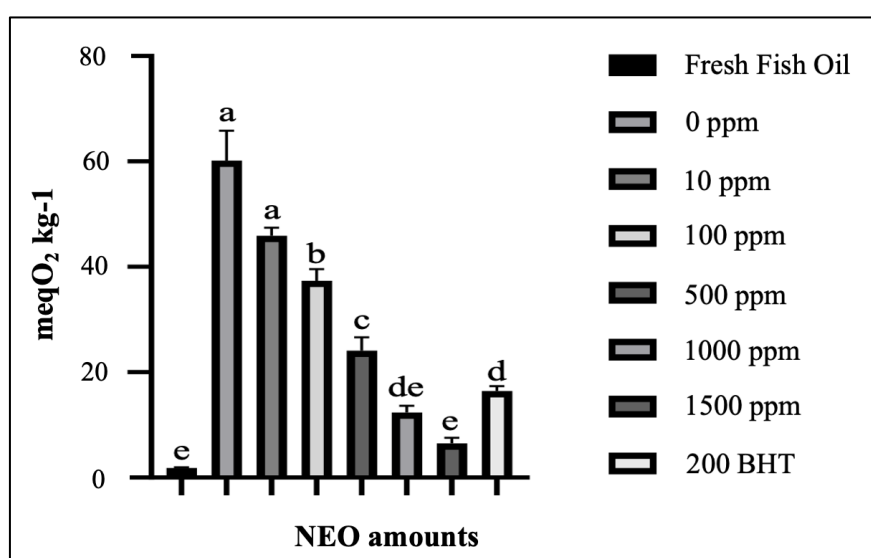
The activities of commercial antioxidants and NEO at different concentrations are also presented in Table 2.

**Table 2.** DPPH scavenging activity of NEO and commercial antioxidants at different concentrations.

Antioxidant	Ppm	DPPH radical scavenging activity (%)
NEO	1000	54.3±2.9 <sup>a</sup>
	500	23.8±4.8 <sup>b</sup>
	250	11.0±4.1 <sup>cd</sup>
BHT	500	52.3±6.7 <sup>a</sup>
	250	26.4±4.6 <sup>b</sup>
	125	13.4±4.2 <sup>c</sup>

NEO: neroli oil; BHT: Butylated Hydroxytoluene; DPPH: 2,2-difenil-1-pikrilhidrazil

Following the rapid oxidation process, the PV values of fish oils with differing amounts of NEO, alongside fish oils without any additives and exposed to thermal oxidation (fresh fish oil), were quantified as seen in Figures 2. The minimum PV value was recorded in fresh fish oil, whereas the maximum PV value was noted in the control group without NEO (0%) ( $p < 0.05$ ). As a consequence of the quick oxidation technique comprising continuous illumination for 48 hours at a constant temperature of 55.0°C and 70% humidity, it revealed that PV generation should be controlled by the use of NEO. Significant variations in PV values were obtained in the experimental groups ( $p < 0.05$ ), with the lowest PV values recorded in fish oils supplemented with 1500 ppm NEO ( $p < 0.05$ ).



**Figure 2.** Peroxide values of fish oils with differing amounts of NEO (n = 3). Values with different letters indicate significant differences between their group ( $p < 0.05$ ).



## Discussion

Research on the extraction of essential oils from various components (such as bark, leaves, and flowers) of Citrus species and their prospective applications is prevalent (Dosoky & Setzer, 2018). Research has established that vital resources derived from citrus sources enhance the physiological processes of fish (Acar et al., 2019; Kesbiç et al., 2020; Acar et al., 2021), livestock (Afzalani et al., 2015), and humans (Navarra et al., 2015). Essential oils derived from Citrus species possess numerous potential benefits (Palazzolo et al., 2013), with its antioxidant efficacy being described as their most notable attribute. D-limonene is typically the predominant constituent of essential oils derived from citrus sources (Ambrosio et al., 2021). While D-limonene alone exhibits limited antioxidant activity (Shah and Mehta 2018), citrus essential oils demonstrate significant antioxidant properties (Ambrosio et al., 2021). The current study uses D-limonene as the major component of the essential oil. However, it also contains high amounts of linalool. Citrus essential oils also contain linalool, a product with high antioxidant activity. Studies have shown that the synergistic effect of D-limonene and linalool improves antioxidant performance (Mirzaei-Najafgholi et al., 2017). The current study's NEO contains 3.50% d-limonene and 25.20% linalool. The presence of these two molecules together in NEO is believed to contribute to its antioxidant performance. The current study determined the IC<sub>50</sub> value of NEO to be 936.31 ppm. In another study, the IC<sub>50</sub> value of NEO was found to be 1040 ± 0.9 ppm (Majnooni et al., 2012). Hacib et al., 2024 determined the essential oil from the peel of orange, another citrus species, to have a value of 903 ppm. It is likely that the different bioactive components in different essential oils and their antagonistic/synergistic effects account for the differences in antioxidant activities.

Fish oil comprises long-chain polyunsaturated fatty acids, rendering it the most effective source for improving the fatty acid profiles of food and feed sources (Pike & Jackson, 2010). However, the global climate issue is increasingly jeopardizing fish oil production and access to this crucial resource. Pelagic fish, including anchovies and sardines, generally produce fish oil. Nonetheless, rising ocean temperatures, excessive fishing, and alterations in ecosystems are depleting populations of these species and complicating sustainable production. In instance, rising sea surface temperatures are affecting the geographical distribution and reproductive behaviors of fish, decreasing fish oil production (Brander, 2007). Moreover, ocean acidification and diminishing oxygen levels resulting from climate change impact fish population survival rates and complicate access to fish oil (Miller et al., 2015). Moreover, the rising demand for fish oil necessitates the creation of sustainable alternatives, particularly for nutritional supplements and aquaculture feeds. Nonetheless, prevailing production costs and supply chain pressures render the provision of fish oil more economically burdensome, particularly in low-income areas (Jackson, 2006). These issues underscore the necessity of sustainable fisheries strategies, the utilization of alternative oil sources, and the efficient management of current resources (Barange et al., 2018; FAO, 2022).

Oxidation represents a significant barrier to the efficient utilization of available resources (Kaitaranta, 1992). PUFA-rich sources are known to be prone to oxidation, with fish oil being a significant source of PUFAs (Phung et al., 2020). To protect fish oil against oxidation, people have long used physical methods like encapsulation or chemical methods like antioxidant additives. Due to the high cost of the encapsulation method, the use of antioxidants is quite common, especially in feed grade oils (Jamshidi et al., 2020). The detrimental effects of synthetic antioxidants have restricted their application (OJEU, 2003). The pursuit of natural antioxidant sources to mitigate oxidation has garnered heightened interest. Lipid oxidation creates both primary and secondary products, with the peroxide value (PV) being the main marker for assessing primary oxidation (Abeyrathne et al., 2021). This study measured the PV

of fresh fish oil at  $1.83 \pm 0.13$  meq O<sub>2</sub>/kg, which falls within acceptable limits for use in fish feed (Korkut et al., 2007). After conducting oxidation tests, it became clear that supplementing NEO reduce peroxide formation in the oil. These findings align with earlier research that highlights essential oils' role in slowing lipid oxidation. For example, in a study with *Apium graveolens* leaf essential oil, heating fish oil at 70°C for 24 hours showed much lower PV levels in samples with essential oil compared to those without it (Kesbiç, 2023). A study analyzing fish oils supplemented with *Borago officinalis* leaf essential oil found that the essential oil supplementation inhibited the formation of PV during the thermal processing of fish oil (Hasdemir et al., 2023).

### Conclusion

In conclusion, NEO can serve as a substitute for BHT, a synthetic antioxidant, owing to its bioactive compounds, including D-Limonene and Linalool. Future research should comprehensively investigate the prolonged and secure antioxidant effectiveness of NEO by assessing the impacts of elevated dosages and secondary oxidation by-products. Furthermore, in vivo research is crucial to confirm the antioxidant effects of NEO in future studies and to explore its potential medical and practical applications in many biological systems.

### Ethical approval

Not applicable

### Informed consent

Not available

### Data availability statement

The authors declare that data can be provided by corresponding author upon reasonable request.

### Conflicts of interest

There is no conflict of interests for publishing this study.

### Funding organizations

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### Contribution of authors

Osman Sabri KESBİÇ: Project administration, Resources, Supervision, Validation, Visualization, Writing original draft, Review, Editing

Hilal METİN: Methodology, Formal analysis, Writing original draft, Software

Ümit ACAR: Conceptualization, Data curation, Writing original draft

All authors have read and agreed to the published version of the manuscript.

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