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GC–MS Analysis and Investigation of Bioactive Potential of Essential Oil from *Citrus aurantium var. amara*

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Abstract: The need for efficient plant-based remedies is becoming acknowledged across the world as the incidence of infectious diseases rises. Essential oils have been actively studied in the research for plant-derived biomolecules that can substitute synthetic medications and, to some extent, eliminate or lessen their side effects. The goal of this study was to explore the presence of phytochemicals in neroli essential oil as well as its many therapeutic properties. We aimed to present a thorough viewpoint on researching essential oil extracted from the bloom of the bitter orange tree as a suitable replacement for synthetic counterparts. The various components present in the essential oil were identified using GC-MS analysis. The oil's antimicrobial properties were tested against a variety of fungal strains. The antioxidant potential was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazy) reagent in a free radical scavenging experiment. The oil exhibited a variable degree of anti-microbial nature against the bacterial and fungal strains. Furthermore, the antimalarial and antituberculosis property was also evaluated against *Plasmodium falciparum* and *Mycobacterium tuberculosis* respectively. Finally, a study for determining the cytotoxicity activity of the oil was studied on the Chinese Hamster Ovary (CHO) cell line. The study's findings revealed that neroli essential oil has natural components that can be used to cure a variety of ailments.

Keywords: Anti-microbial, Anti-malarial, Anti-tuberculosis, Antioxidant, Cytotoxicity, DNA Barcoding, GC-MS

1. Introduction

Citrus aurantium var. amara (Rutaceae family) is one of the oldest, most traded, and most consumed crops [1]. Its cultivation is documented as far back as 2100 BC. Citrus's origins are still debated; however, it is thought to have originated in Southeast Asia [2]. It's also known as sour orange or bitter orange, and it has a slew of benefits, including resistance to a variety of viral infections, cold tolerance, and an improvement in the fruit quality of grafted plants. Because of the abundant bioactive substances including as phenolics, flavonoids, essential oils, and vitamins, it is also one of the species that has been used for medical purposes [3]. Fruit peels, flowers, and plant leaves are the parts most commonly utilized for therapeutic purposes.

Citrus aurantium var. amara is used to treat a range of diseases as an alternative medicine. Some studies reveal the effect of *Citrus aurantium var. amara* as a preoperative anxiolytic drug [4]. It has been shown to have anti-anxiety effects in rats through influencing 5-HT receptors [2]. It also shows skin healing properties and is used to reduce acne as well as eczema [5]. The essential oil of bitter orange is used as an anticonvulsant agent. It's been used to treat seizures and epilepsy [5]. Neroli EO is a smooth-muscle and endothelium-dependent vasodilator that can help with cardiovascular symptoms. The endothelial component is mediated by the nitric oxide to

solubilize guanylyl cyclase pathway, whereas the smooth muscle component is mediated by the ryanodine receptor (RyR) signaling pathway, which involves blocking extracellular Ca^{2+} influx and store operated Ca^{2+} release [6]. A study conducted on university students, revealed the oils' ability to reduce the symptoms of premenstrual syndrome (PMS) [7]. In addition to its anti-depressant properties, it has favorable benefits on mood, blood pressure, pain, inflammation, bloating, and indigestion [7]. Furthermore, Neroli EO is a strong antioxidant. Studies have revealed a 100% singlet oxygen scavenging activity at all concentrations between 0.1 and 2% [8].

Despite its numerous benefits, *Citrus aurantium var. amara* has received only a limited amount of research. As a result, the purpose of our research is to assess the biological activities of essential oils and their constituents, such as anti-malarial, antibacterial, and antifungal qualities. Agar well diffusion and the Agar well technique was used to test *Citrus aurantium var. amara* against gram-positive and gram-negative bacteria, and the Minimum Inhibitory Concentration was obtained. The oil was also tested against *P. falciparum* strains as well as fungus from the Ascomycetes and Mucormycota families.

2. Methodology

2.1. Sample Collection, Authentication, and Extraction of NEO

The blossoms of Bitter orange plant (*Citrus aurantium var. amara*) were collected from Solangapalayam, Erode, (11°13'00.4"N 77°50'19.2"E) Tamil Nadu. It was then brought to the research centre. The obtained sample was validated using the DNA barcoding technique published by [9]. The sample's genomic DNA was isolated using CTAB buffer. By using the gel electrophoresis technology, the quantitative analysis of the collected genome was performed. The rbcL universal primers (Forward 5'-ATGTCACCACAAACAGAG-3' and Reverse 5'-GTAAAATCAAGTCCACCRCG-3') were used to amplify the genomic DNA sample using a polymerase chain reaction (PCR) Thermocycler. Following that, the rbcL gene was sequenced using Sanger sequencing methodology at Eurofins laboratory in India. Then the sequencing data was submitted to the National Center for Biotechnology Information (NCBI). Finally, the obtained DNA sequence of *Citrus aurantium var. amara* with the GenBank accession number of OL804186 was compared to all known rbcL gene sequences of *Citrus aurantium* in the NCBI database to determine their phylogenetic relationship. The sequences were aligned using the MEGA 11 computational tool, and a phylogenetic tree was constructed using the UPGMA algorithm with bootstrap values based on 1000 replications and the Tamura-Nei model [10].

For oil extraction blossoms of Bitter orange plant were washed to get rid of any dirt particles settled on the flowers. Before beginning with the extraction process, the petals were removed from the sepals, weighed, and air-dried at room temperature. The essential oil was extracted using a Clevenger-type apparatus and hydro distillation method. Any traces of residual moisture present in the extracted oil were eliminated by using anhydrous sodium sulphate.

2.2. GC-MS Analysis

The analysis conducted, utilized a Shimadzu GCMS-QP2010 system, which encompassed a Gas Chromatograph associated with a Mass Spectrometer and an Rtx-5ms column with 5% phenyl-methylpolysiloxane composition, a 30 m long capillary column with a 0.25 mm internal diameter, and a 0.25 mm film thickness [11]. An electron ionization device in electron impact mode with a 70-eV ionization energy was used in the GC-MS technique. The operation employed helium gas (99.999%) as the transporter gas, with a uniform purge flow of 3.0 mL/min [12]. Considering the temperature regulation, the injector temperature and ion-source temperature were held at 250°C and 220°C, respectively, while the oven temperature was kept at 40°C for 3 mins; and was then customized to 120°C for 3 mins. In this manner, the temperature was raised to 180°C and held for 3 mins, again held for 5 mins followed by a post-run at 230°C for 3 mins. The entire analytical procedure took 65 mins. The mass spectrometer was balanced at an electron multiplier voltage of 1.61 kV. The start mass to charge ratio was 40.00, while the end mass to charge ratio was 1000.00. For analysis purposes, the essential oil was dissolved in DMSO. The rate organization of the fundamental oil was processed by the standardization strategy from the GC top peaks. The results were calculated as the average of two infusions.

2.3. Microbial Strains Used in the Study

The antibacterial properties of the test specimens were assessed against nine bacterial strains and three fungal strains. *Enterococcus hirae, Propionibacterium acnes, Proteus mirabilis, Staphylococcus epidermidis, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, and Mycobacterium tuberculosis* which served as the bacterial strains used in this study. The fungal strains used utilized were *Aspergillus niger, Botritis,* and *Candida albicans*. Some strains were procured from the National Centre for Cell Science (NCCS), Pune, whereas some were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, and the rest of the bacterial and fungal cultures were obtained from existing laboratory test isolates. For experimental purposes, active cultures were developed by inoculating the loopful of cells from glycerol stock culture to flasks containing sterilized Luria Bertani (LB) broth followed by incubation at 37°C for 24 hours. The fungi were cultivated in Sabouraud Dextrose Broth and were incubated for 24 hours at 37°C.

2.4. Anti-microbial Analysis

2.4.1. Antibacterial Activity of NEO

The bacterial cultures that had been cultivated overnight at 30°C in sterile Luria-Bertani (LB) broth were adjusted to 1.0×105 CFU/mL. Then 2 mL suspension culture was spread

plated on top of each petri plate with about 6 mL Luria-Bertani Agar (LA). Further, one sterile filter disc (6 mm) was placed on top of each plate and 10 µL of the test sample was added to each disc, with Kanamycin (30 µg/mL) serving as a positive control. The plates were then examined to determine the zone of clearance after a 24-hour incubation period at 37°C [13].

2.4.2. Evaluation of Antifungal Activity of NEO

NEO was screened for its antifungal activity against three fungal strains namely *Aspergillus niger*, *Botrytis*, and *Candida albicans*. Initially, the fungal culture (adjusted to 1.0×106 CFU/mL) was spread out onto Sabouraud Dextrose Agar-coated petri plates (SDA). On the agar plates, two sterile filter discs with a diameter of 6 mm were put, followed by 10 µL of NEO and Ketoconazole (1 mg/mL) which served as a positive control. For a period of 3-7 days, the plate was inoculated at 37°C. The plates were observed for clearance areas, and their diameters were measured [14].

2.4.3. Evaluation of Minimal Inhibitory Concentration (MIC) for Bacteria

The Minimum Inhibitory Concentration (MIC) of the bacterial strains was assessed by Resazurin Microtiter Assay (REMA). A 96-well microtiter plate with a flat bottom was used for this test. A two-fold serial dilution in 1X Mueller Hinton Broth was done, from 1000g/mL to 1.952 g/mL. 100 µL of bacterial culture was added to each well (adjusted to 105-106 CFU/mL). The cultured microplates were sealed lid and incubated at 37°C for 24 hours. The MIC of samples was detected after the addition (5µL) of 2mg/mL and an incubation period of 30 mins at 37°C. Microbial growth was determined by observing the color shift of Resazurin in the microplate wells. The MIC was defined as the lowest concentration of sample that caused no color shift [15].

2.4.4. Evaluation of Minimal Fungal Concentration (MFC) for Fungi

The fungal strains were tested against NEO to evaluate its Minimum Inhibitory Concentration (MIC) value. The experiment was carried out in a 96-well microtiter plate wherein the first two columns of each row were treated as positive and negative control. Ketoconazole (1mg/mL) served as a positive control. The test was carried out using a twofold serial dilution procedure in 1X Mueller Hinton broth, with a starting oil content of 1000 µg/mL and increasing it to 1.953 µg/mL. Each well received a 100 µL amount of fungal culture at 105-106 CFU/mL, and all wells were properly mixed. After that, the test microtiter plate was incubated at 37°C for 24 hours. After the incubation period, 5µL of Resazurin dye (2mg/mL) was added to each well to observe the microbial growth due to the emergence of pink color. The lowest inhibitory concentration was noted in the well consisting of minimum oil concentration, which showed no change in color [16].

2.4.5. Assessment of Anti-mycobacterial Activity of Minimum Inhibitory Concentration (MIC) Assay

The anti-mycobacterial activity of NEO was evaluated using the [17] technique in the REMA assay. In DMSO, a working stock of oil was generated with a concentration of 100 mg/mL. The *mycobacterium* culture was cultivated for 20 days in Middlebrook 7H9 broth base medium supplemented with oleic acid, albumin, dextrose, and catalase (OADC) Enrichment and 0.2% glycerol. After sufficient growth was observed, the culture was adjusted to 1.0×105 CFU/mL followed by which necessary volume of culture was introduced in each McCartney glass tube. Then, in each tube, NEO was introduced at varying concentrations ranging from 100 µg/mL to 1000 µg/mL. The tubes were kept in a CO_2 incubator at 37°C for 28 days. Following the incubation time, 100 µL of the incubated mixture was added to each microtiter well, along with 25 µL of 0.4 mg/mL Resazurin, to observe the microbial growth through a color shift from colorless to pink. The lowest inhibitory concentration against *Mycobacterium tuberculosis* was recorded in the well which showed no color change.

2.4.6. Antioxidant Analysis via DPPH (2,2-diphenyl-1-picrylhydrazy) Assay

The free radical scavenging potency of NEO was studied in the free radical 2,2-diphenyl-1-picrylhydrazy (DPPH) assay. An oil stock of concentration 10 mg/mL was prepared with varying concentrations ranging from 0.1 mg/mL to 1 mg/mL in dimethyl sulfoxide (DMSO). Each tube had a total reaction volume of 4 mL, comprising of the sample, methanol as a diluent, and 2 mL of DDPH. The reaction mixture was incubated for 30-45 mins in a dark room. The absorbance value was then measured at 515 nm using the UV Visible spectrophotometer (Lab India UV 3000+).

The % inhibition of the DPPH radical for each concentration was calculated using the following formula:

$$\text{Percentage Free Radical Scavenging Activity} = \frac{\text{Absorbance}_{\text{blank}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{blank}}} \times 100$$

2.4.7. In-vitro Antimalarial Analysis

This in-vitro assay was performed in a 96-well microtiter plate as per the micro assay protocol of [18] micro assay technique with some modifications. The drug-resistant and sensitive strains of *Plasmodium falciparum* were cultured and stored in the Roswell Park Memorial Institute (RPMI) 1640 supplemented with 25 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% D-glucose, 0.23% sodium bicarbonate and 10% heat-inactivated human serum [19].

The non-synchronized parasites of *P. falciparum* were transitioned to synchronized using 5% D-sorbitol treatment to obtain the ring stage parasitized cells only. Initially, an initial ring stage parasitemia of 0.8 to 1.5% at 3% haematocrit was determined by Jaswant Singh Bhattacharya (JSB) staining in a total volume of 200 µL of medium RPMI-1640 to assess the % parasitemia (rings) and uniformly maintained with 50% RBCs (O+) for carrying out the assay. Each of the test samples had a stock solution of 5 mg/mL produced in DMSO, and further dilutions were made with culture media. In duplicate wells and test wells, which

contained the parasitized cell preparation, diluted samples in 20 µL volume were added to produce the final concentrations (at fivefold dilutions) ranging from 0.4 µg/mL to 100 µg/mL. The plates were incubated at 37°C for 36-40 hours. Thin blood smears from each well were prepared and stained with JSB stain after 36 to 40 hours of incubation. The lowest inhibitory concentrations were defined as the test concentrations that inhibited complete maturation to schizonts and trophozoites (MIC). Chloroquine and Quinine were the reference drugs used in this study.

2.4.8. Antituberculosis Activity Evaluation

The NEO was screened in vitro for its activity against a strain of *Mycobacterium tuberculosis* (H37Rv) which was procured from the National Institute for Research in Tuberculosis, Chennai. The EO was tested using the [20] method. The L.J medium used was enriched with salts, Malachite green, 2% solution, Asparagine, Glycerol, and other growth requirements. Being an egg-based media, it comprises of homogenized egg solution which was made from fresh hen's eggs. Isoniazid was utilized as a reference drug in this study. The media had varying concentrations of NEO (100, 50, 12.5, 6.25. 3.125, 10, 5, 2.5, 1.25, 8, 4, 2, 1, 0.5, 0.25 µg/mL) which were then inoculated with the Mycobacterium strain. Incubation was carried out and weekly observations were noted.

2.4.9. Determination of Cytotoxic Activity Against CHO Cell Line

MTT assay was used to screen the cytotoxic potential of NEO. A 96-well microplate was used to seed the cells at a density of 5×104 cells/well. It was then incubated at 37°C, 5% CO_2 overnight. After observation, the completely merged cells were treated with the essential oil samples at varying concentrations and were then kept for incubation. The cells were observed, and 10 µL of 5 mg/mL MTT reagent was introduced, followed by incubation for 4 hours. The spent media along with the sample was discarded, 100 µL of DMSO was added to each well and the plate was then incubated on a shaker for 10 mins for the dissolution of formazan crystals. Finally, the absorbance of each well was observed at 570 nm wavelength in a 96-well ELISA plate-reader.

The cell inhibition % age was calculated using the following formula [21]:

$$\% \text{ Inhibition} = \left[\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}\right] \times 100$$

The IC_{50} value was quantified using graph tools.

3. Result and Discussion

3.1. Authentication and Extraction of NEO

The DNA barcoding strategy was utilized to verify the oil obtained from the blossoms of *Citrus aurantium var. amara*, as this technology has been employed in the field of taxonomy and as a valuable tool for quality control and therapeutic safety. This approach has recently gained popularity in areas such as cryptic plant species identification, phylogenetic study, culture diversity conservation, and traditional medical plant authentication [22].

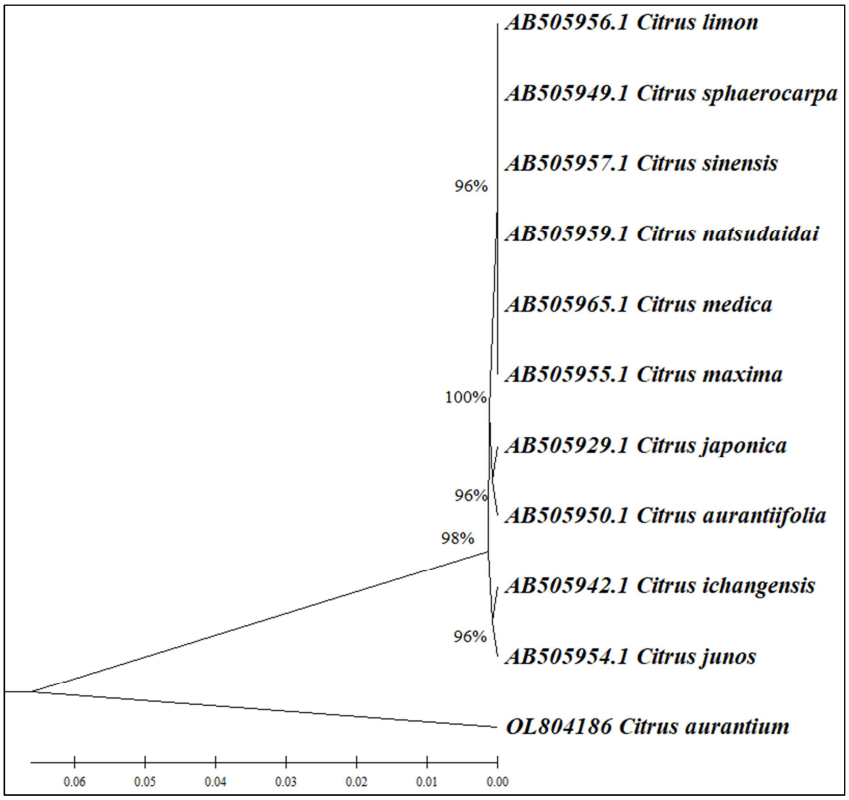

Figure 1. *Phylogenetic tree of Citrus aurantium var. amara with different species of Citrus.*

The gathered plant sample's rbcL gene was sequenced and compared to some known rbcL gene sequences of Citrus species in the NCBI database, revealing that it is closely related to the genus *Citrus*. As a result, its phylogenetic analysis revealed that it is closely linked to different species of *Citrus* with a 98% similarity to all the species of *Citrus* genus namely *Citrus maxima, Citrus ichangensis, Citrus japonica, Citrus medica, Citrus natsudaidai, Citrus sinensis, Citrus limon, Citrus junos, Citrus aurantiifolia,* and *Citrus sphaerocarpa*. The MEGA 11 computational tool was used to create a UPGMA phylogenetic tree for *Citrus aurantium var. amara*, demonstrating that it belongs to the *Citrus* genus, as shown in Figure 1.

3.2. Determination of Chemical Constituents by GC-MS Analysis

The chromatographic profile was obtained by employing GC-MS technique wherein 17 components were identified including monoterpenes, sesquiterpenoids, sesquiterpenes, diester of pthalic acid, and isoprenoids. The analysis revealed 1-Ethanol, 2-(ethylsulfinyl)- (93.84%) as the principal compound present in the essential oil followed by β.-Pinene (0.38%), 3-Carene (0.22%), D-Limonene (0.12%), Eucalyptol (0.1%), 1,6-Octadien-3-ol, 3,7-dimethyl- (0.69%), L-.α.-Terpineol (0.31%), 2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)- (0.17%), 1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate (0.34%), and (R)-lavandulyl acetate (0.08%) which were the top 10 components of the oil. The retention time of these components was 7.5, 8.898, 10.012, 10.633, 10.724, 13.432, 15.973, 17.049, 17.75, and 21.133 mins respectively (Table 1). However, the above-mentioned GC-MS profile of the oil varies remarkable when compared to that of [23].

Table 1. *Components of Neroli revealed with the help of GC-MS analysis.*

Component Name	Component composition (%)	Retention time (min)	Height
1-Ethanol, 2-(ethylsulfinyl)-	93.84	7.5	79351410
.beta.-Pinene	0.38	8.898	18106357
3-Carene	0.22	10.012	9810052
D-Limonene	0.12	10.633	5610224
Eucalyptol	0.1	10.724	4686864
1,6-Octadien-3-ol, 3,7-dimethyl-	0.69	13.432	23142139
L-.alpha.-Terpineol	0.31	15.973	10399600
2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	0.17	17.049	4649678
1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate	0.34	17.75	9414992
(R)-lavandulyl acetate	0.08	21.133	1387940
2,6-Octadien-1-ol, 3,7-dimethyl-, acetate	0.14	21.898	2811508
Nerolidyl acetate	0.04	28.658	839965
Nerolidyl acetate	0.06	30.138	1182867
Diethyl Phthalate	3.47	32.098	27720971
.beta.-Bisabolene	0.02	37.134	205545
(E)-.beta.-Famesene	0.02	38.369	188584

3.3. Anti-microbial Activity Analysis

3.3.1. Determination of Antibacterial Activity

The antibacterial activity of NEO was tested using the paper disc diffusion method against several gram-positive and gram-negative bacteria strains. Under undiluted concentrations, the essential oil showed varying degrees of antibacterial action against the identified microbiological pathogens, as shown in (Table 2). Depending on the outcomes, the best activity was shown against *Klebsiella pneumoniae*, with an inhibitory zone of 13.05 ± 0.09 mm. Other bacterial strains such as *Enterococcus hirae* (10.01 ± 0.08mm), *Propionibacterium acnes* (12.03 ± 0.06 mm), *Proteus mirabilis* (10.04 ± 0.07 mm), *Staphylococcus epidermidis* (12.01 ± 0.04 mm), and *Escherichia coli* (13.03 ± 0.07 mm) exhibited similar results except *Streptococcus pneumoniae*, which exhibited no zone of inhibition (Figure 2). The antibacterial property might have been resulted due to presence of antibacterial compounds such as β-Pinene [24], Eucalyptol [25], D-Limonene [26], 1,6-Octadien-3-ol,3,7-dimethyl- [27], L-.α.-Terpineol [28], and Nerolidyl acetate [29] which were revealed by GC-MS analysis. Due to these remarkable results and the presence of multiple antibacterial components, NEO can be considered as a highly potent antibacterial agent against the pathogenic bacterial strains in focus.

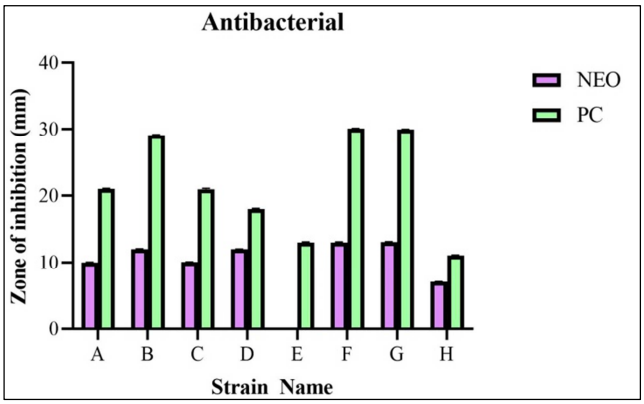

Figure 2. *Antibacterial activity of NEO against (A) Enterococcus hirae, (B) Propionibacterium acnes, (C) Proteus Mirabilis, (D) Staphylococcus epidermidis, (E) Streptococcus pneumoniae, (F) Escherichia coli, (G) Klebsiella pneumoniae, and (H) Pseudomonas aerugino.*

Table 2. *Antibacterial activity of NEO against the bacterial strains used.*

Anti-bacterial		
Strain Name	**Zone of inhibition (mm)**	
	Positive Control	**Microbial Strain**
Enterococcus hirae	21.04 ± 0.04	10.01 ± 0.08
Propionibacterium acnes	29.1 ± 0.06	12.03 ± 0.06
Proteus Mirabilis	20.99 ± 0.08	10.04 ± 0.07
Staphylococcus epidermidis	18 ± 0.06	12.01 ± 0.04
Streptococcus pneumoniae	13 ± 0.06	0
Escherichia coli	30.08 ± 0.05	13.03 ± 0.07
Klebsiella pneumoniae	29.97 ± 0.04	13.05 ± 0.09
Pseudomonas aeruginosa	11.05 ± 0.06	7.03 ± 0.08

3.3.2. Determination of Antifungal Activity

Considering the presence of the components β-Pinene [24], and 1,6-Octadien-3-ol, 3,7-dimethyl- [27] in NEO, the anti-microbial results of NEO against the test fungal strains exhibited considerable fungal activity. Three fungal strains- *Aspergillus niger, Botrytis* and *Candida albicans*, were used in this study, as mentioned in (Table 3). The highest zone of inhibition was observed against *Aspergillus niger* (17.98 ± 0.05 mm) followed by *Candida albicans* (17.05 ± 0.05 mm) (Figure 3). The results obtained in the study are in conformity with those published by [30] and [31] wherein, different essential oils consisting of the same component were studied for their anti-fungal property.

Table 2. *Antifungal activity of NEO against Fungal strains used.*

Antifungal		
Strain Name	**Zone of inhibition (mm)**	
	Positive Control	**Values**
Aspergillus niger	18.97 ± 0.04	17.98 ± 0.05
Botrytis	18 ± 0.06	16.05 ± 0.06
Candida Albicans	16.03 ± 0.04	17.05 ± 0.05

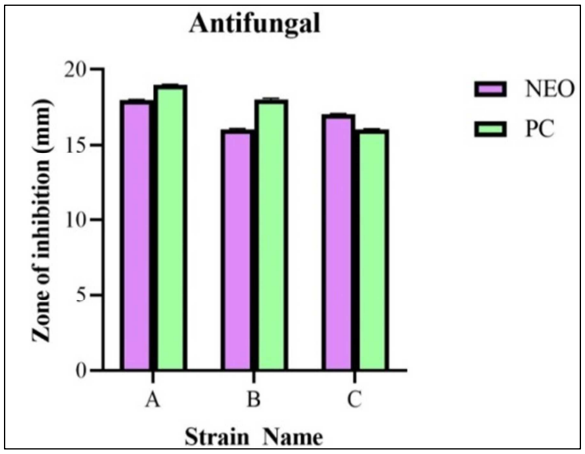

Figure 3. *Antifungal activity of NEO against (A) Aspergillus niger, (B) Botrytis, and (C) Candida Albicans.*

3.3.3. Determination of Minimum Inhibitory Concentration

MIC data of NEO is illustrated in (Table 4). Based on the results obtained, the essential oil successfully inhibited the growth of all the test microbial strains. The results were found to be remarkable in case of gram-negative bacterias *Proteus mirabilis* (333.33 ± 144.34 µg/mL), *Enterococcus hirae* (291.67 ± 190.94 µg/mL) and *Pseudomonas aeruginosa* (208.33 ± 72.17 µg/mL). These values indicate the resistance potential of the microorganisms against NEO at higher concentrations (Figure 4). The activity of NEO against *Mycobacterium tuberculosis* (H37Rv) was observed at a MIC value of 50 µg/mL.

Table 4. *MIC value of NEO against Bacterial strains.*

MIC	
Strain Name	**Values**
Enterococcus hirae	291.67 ± 190.94
Propionibacterium acnes	145.83 ± 95.47
Proteus Mirabilis	333.33 ± 144.34
Staphylococcus epidermidis	72.92 ± 47.74
Streptococcus pneumoniae	0
Escherichia coli	36.46 ±23.87
Klebsiella pneumoniae	72.92 ± 47.74
Pseudomonas aeruginosa	208.33 ± 72.17
Mycobacterium Tuberculosis	50.33 ± 0.48

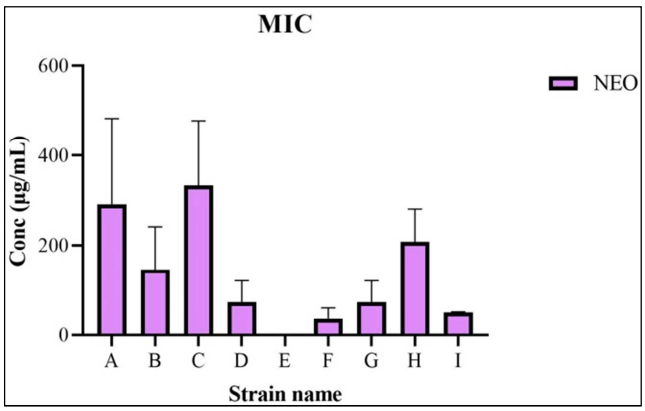

Figure 4. *Graph representing MIC value of NEO against (A) Enterococcus hirae, (B) Propionibacterium acnes, (C) Proteus Mirabilis, (D) Staphylococcus epidermidis, (E) Streptococcus pneumoniae, (F) Escherichia coli, (G) Klebsiella pneumoniae, (H) Pseudomonas aeruginosa, and (I) Mycobacterium Tuberculosis.*

3.3.4. Minimal Fungal Concentration (MFC) for Fungi

The values obtained for minimum inhibitory concentration for fungi of NEO has been summarized in (Table 5). The MFC value was highest for *Aspergillus niger* (36.46 ± 23.87 µg/mL), followed by *Botrytis* (26.04 ± 9.02 µg/mL) and *Candida albicans* (18.23 ± 11.93 µg/mL), which is displayed in the form of a graphical representation in (Figure 5).

Table 5. *MFC value of NEO against Fungal Strains.*

MFC	
Strain Name	**Values**
A. Niger	36.46 ± 23.87
Botrytis	26.04 ± 9.02
Candida Albicans	18.23 ± 11.93

Table 6. *Percentage scavenging activity of NEO.*

Antioxidant		
Concentration (mg/mL)	**Absorbance measured at 515 nm**	**Free Radical Scavenging Activity (%)**
Control (DPPH)	0.725	
0.1	0.593	18.14 ± 0.06
0.2	0.553	23.7 ± 0.07
0.3	0.521	28.14 ± 0.05
0.4	0.473	34.8 ± 0.06
0.5	0.427	41.14 ± 0.08
0.6	0.414	42.93 ± 0.04
0.7	0.325	55.11 ± 0.08
0.8	0.288	60.23 ± 0.06
0.9	0.211	70.88 ± 0.07
1	0.131	81.91 ± 0.07

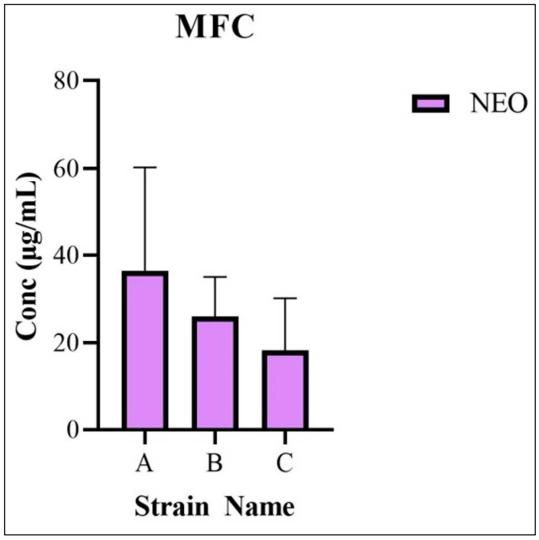

Figure 5. *Graph representing MFC value of NEO against (A) Aspergillus niger, (B) Botrytis, and (C) Candida Albicans.*

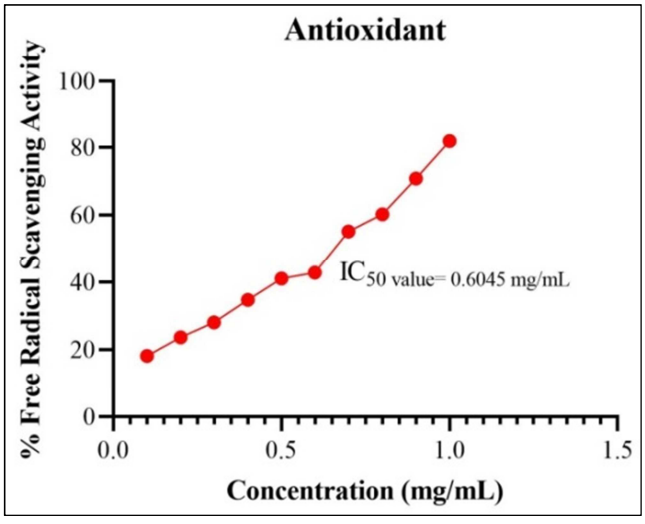

Figure 6. *Percentage Free Radical Scavenging Activity of NEO.*

3.3.5. Antioxidant Analysis of NEO

The efficacy of NEO to scavenge free radicals was investigated in this research using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. Free radicals were scavenged by the essential oil's inherent antioxidant activity in a dose-dependent manner because of the presence of certain components such as D-Limonene [32], L-.α.-Terpineol [33], and Nerolidyl acetate [34] which resulted in percentage inhibition as shown in (Table 6). The lowest free radical scavenging activity was reported at 0.1 mg/mL with a percentage inhibition of 18.14 ± 0.06 mg/mL and the highest value of 81.91 ± 0.07 mg/mL was reported at 1 mg/mL. The oil reported an IC50 value of 0.6045 mg/mL as seen in (Figure 6). The IC50 value of the oil was found to be significantly different from the one reported by [35].

3.3.6. Anti-Malarial Potential of NEO

Malaria is one of the most serious diseases spread by Anopheles mosquitoes, and it is a major public health issue in many underdeveloped nations [36]. The antimalarial activity of the essential oil was tested against drug-sensitive and drug-resistant *Plasmodium falciparum* strains using the Minimum Inhibitory Concentration (MIC) technique. The IC50 values of NEO drug-sensitive and drug-resistant *P. falciparum* were found to be 0.40 µg/mL and 0.97 µg/mL respectively (Table 7). (Figure 7) depicts survival vs. oil concentrations ranging from 0.1 to 2 µg/mL visually. As per our knowledge, this is the first study where the antimalarial potential of NEO was accessed. However, the components possessed by the oil which are accountable for this antimalarial characteristic need to be studied.

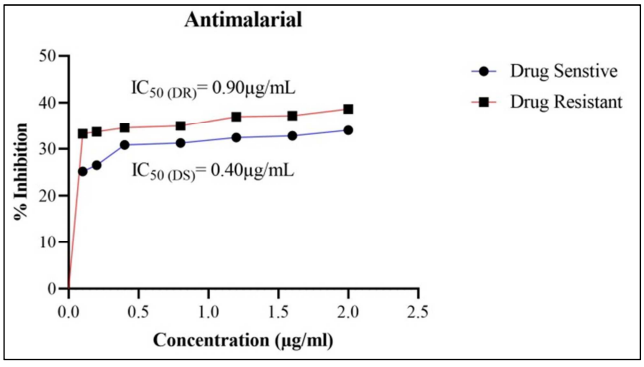

Figure 7. *Antimalarial activity of NEO against drug resistant and drug sensitive strains of Plasmodium falciparum.*

Table 7. *Antimalarial Activity of NEO against Plasmodium falciparum.*

Antimalarial				
NEO	**^{DS}*Plasmodium falciparum***		**^{DR}*Plasmodium falciparum***	
Concentration (µg/ml)	**% Inhibition**	**IC50 value**	**% Inhibition**	**IC50 value**
0.1	25.19		33.25	
0.2	26.5		33.68	
0.4	30.85		34.53	
0.8	31.25	0.40 µg/ml	34.98	0.97 µg/ml
1.2	32.44		36.99	
1.6	32.8		37.22	
2	34		38.68	
Standard Chloroquine	0.020 mg/ml			
Standard Quinine	0.268 mg/ml			

3.3.7. Cytotoxic Potential to Inhibit Cell Lines

The cytotoxic potential of NEO was determined using MTT assay against CHO a non-cancerous cell line with different concentrations of 5, 10, 20, 40, 60, 80 and 100 µg/mL. The IC50 value of this assay was found to be 43.35 µg/mL. Table 8 and Figure 8 show the percentage inhibition results as well as the curve indicating NEO cytotoxic activity against the CHO cell line.

Table 8. *Percentage inhibition of NEO against CHO cell line.*

Cytotoxicity			
Concentration (µg/mL)	**% Viability**	**% Inhibition**	**Values**
5	76.71517672	23.28482328	22.98 ± 0.04
10	77.23492723	22.76507277	24.21 ± 0.06
20	74.22037422	25.77962578	30.82 ± 0.05
40	64.03326403	35.96673597	41.62 ± 0.06
60	52.7027027	47.2972973	47.54 ± 0.06
80	52.28690229	47.71309771	51.01 ± 0.05
100	45.73804574	54.26195426	54.27 ± 0.07

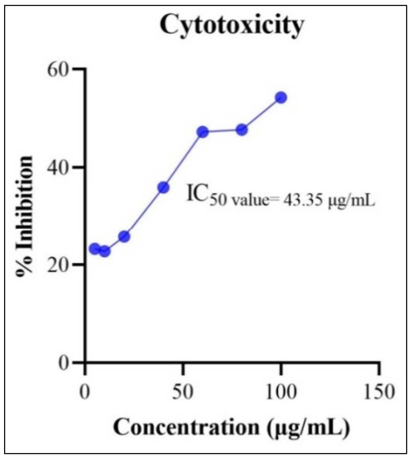

Figure 8. *Cytotoxicity of NEO against CHO cell line.*

4. Conclusion

Our research looked at the possibility of using *Citrus aurantium var. amara* essential oil as a substitute for synthetic medications that have been linked to negative health effects in the past. The current study delves into the chemical composition of *Citrus aurantium var. amara* essential oil as well as its antibacterial, anti-malarial, antioxidant, antituberculosis, and cytotoxic properties. The oil's GC-MS analysis revealed the presence of certain components which revealed the oil's anti-microbial potency against the test bacteria. They are particularly enticing because they are all-natural and have higher bioactivities than synthetic compounds. To the best of our knowledge, this is the first study to report cytotoxic potential against CHO cell line with promising results, as well as anti-malaria and anti-tuberculosis potential of the oil against two deadly pathogens, *Plasmodium Falciparum* and *Mycobacterium Tuberculosis*. In a summary, due to its diverse activities, Neroli essential oil (*Citrus aurantium var. amara*) has a significant potential to be used as a strong pharmacological substance, and if explored further might be used in a number of sectors to solve different inadequacies. As a result, more study is required to develop natural alternatives to synthetic industrial by-products that may be used in a range of medical, food, cosmeceutical, and nutraceutical applications.

Abbreviations

EO	Essential Oil
NEO	Neroli Essential Oil
CHO	Chinese hamster ovary cell
GC-MS	Gas chromatography–mass spectrometry

5-HT	5-hydroxytryptamine
rbcL	ribulose-1,5-bisphosphate carboxylase large subunit
DPPH	2,2-diphenyl-1-picrylhydrazyl
RyR	Ryanodine receptor
DNA	Deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
PCR	Polymerase chain reaction
NCBI	National Center for Biotechnology Information
MEGA 11	Molecular Evolutionary Genetics Analysis version 11
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
NCCS	National Centre for Cell Science
MTCC	Microbial Type Culture Collection and Gene Bank
LB	Luria-Bertani
CFU	Colony Forming Unit
SDA	Sabouraud Dextrose Agar-coated petri plates
MIC	Minimum Inhibitory Concentration
REMA	Resazurin Microtiter Assay
OADC	Oleic acid, Albumin, Dextrose, and Catalase
CO_2	Carbon dioxide
UV	Ultraviolet
mM	millimolar
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L.J medium	Löwenstein–Jensen medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ELISA	Enzyme-linked immunoassay
MFC	Minimal Fungal Concentration
IC_{50}	half maximal inhibitory concentration

Data Availability Statement

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Author Contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Conflict of Interest

The authors declare that they have no competing interests.

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