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Chemical profiling and *in-vitro* anti-oxidant, anti-diabetic, anti-inflammatory, anti-bacterial and anti-fungal activities of essential oil from *Rosmarinus officinalis* L.

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Abstract

Rosmarinus officinalis L. commonly known as Rosemary is a well-known genus belongs to Lamiaceae family, which is one of the major plant families with a high concentration of essential oils. Rosemary is well known to produce herbal essential oils owing to the presence of therapeutic and medicinal elements in the plant's by-products. In the present study, rosemary essential oil (REO) was extracted from *Rosmarinus officinalis* and it was evaluated for its phytochemicals, antioxidant, antibacterial, antifungal, anti-diabetic and anti-inflammatory activities. GC-FID analysis and fingerprint analysis was performed. Different antioxidant assays were performed like DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2-azinobis-3-ethylbenzothiazoline-6-sulphonic acid), nitric oxide radical, hydroxyl radical, iron reducing potential, iron chelating activity. Anti-bacterial activity was observed by the disk diffusion method against Gram-positive (G^+), Gram-negative (G^-) bacteria. Anti-diabetic and anti-inflammatory activities were also performed. Antifungal activity against Aspergillosis and Mucormycosis causing fungal strains was also evaluated. GC-FID revealed the presence of vertocitral, α-pinene andhumulene oxidemajor component of REO along with other bioactive components. The IC50 value (µl/ml) for various assays was observed in the following order: OH^- (16.6118), iron chelating (32.0115), iron reducing (92.534), DPPH (101.929), and ABTS (172.2765). The best antimicrobial activity was against *Bacillus subtilis (MTCC 121),* with an inhibition zone of 2.5 cm. REO showed strong anti-inflammatory and anti-diabetic potential and can be employed as an anti-diabetic agent due to its inhibitory effect on α-amylase activity. Based on these findings it was concluded that REO can be widely employed across a variety of sectors, including food, aromatherapy, herbal and allopathic medicine, cosmetics, and as a natural biocide, fungicide, and preservative.

Keywords: aspergillosis; anti-diabetic; antioxidant; anti-inflammatory; essential oil; mucormycosis

Introduction

In developing nations, aromatherapy and traditional medicine still heavily rely on aromatic and medicinal herbs. There are now several herbal treatments used in medicine (Bakkali *et al*., 2008). A sensible

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strategy in our search for novel medications is to examine the biology and pharmacological characteristics of medicinal plant extracts. People's and researcher's attention has to be drawn to plant medicines due to their less adverse effects (Fabricant *et al*., 2001). In other words, everyone wants pharmaceuticals and food with less synthetic ingredients (Gyawali *et al*., 2014). Plants produce primary and secondary metabolites, which have a wide range of functions (Wu *et al*., 2016). Chemically complicated secondary metabolites are made up of a variety of compounds which include alkaloids, flavonoids, phenols, essential oil, and saponins. The most significant secondary metabolites of medicinal plants are essential oils derived from plants, which are regarded as green technology (Wu *et al*., 2016). An essential oil is a concentrated hydrophobic liquid containing a mixture of complex volatile chemical bioactive compounds from aromatic plants. Essential oils are, natural, aromatic chemical compounds that retain the natural smell and flavor of their source (Mohamed *et al*., 2023). The characteristic odor and color of EOs depend on the origins of plants, species, and organs. Plant essential oils have different chemical contents based on geographical location, environment, and stage of maturity are all factors that can affect plants and the percentage of essential oils in them (Mohamed *et al*., 2023). These prevent biological systems from experiencing free radical redox reactions and are used by more than half of the world's population as a treatment for a variety of ailments (Fabricant *et al*., 2001). Plant organs like buds, stems, roots, seeds contain large amounts of essential oils. Essential oils have been used for medicinal and therapeutic purposes throughout numerous cultures for thousands of years. Essential oils are recently gaining popularity as natural and cost-effective therapy for a number of health concerns because of antidepressant, stimulating, detoxifying, antibacterial, antiviral and calming properties (Kar*et al*., 2018). In addition to being utilised as food additives and preservatives, essential oils are also used as natural treatments, sanitary goods, cosmetics, perfumes, and make-up products (Bakkali *et al*., 2008). Another biological characteristic of great interest is the antioxidant activity of essential oils, which may protect food from the harmful effects of oxidants. Essential oils are also able to scavenge free radicals and act as anti-inflammatory agent (Miguel, 2010).

Rosmarinus officinalis L. commonly known as Rosemary and belongs to Lamiaceae family. Rosemary spare more than 24 cultivars native to the mediterranean region and is cultivated worldwide including various countries like: Iran, Morocco, Tunisia, Spain, France, India, Italy, Yugoslavia and also in America (Nieto *et al*., 2018; de Macedo *et al*., 2020; Macedo *et al*., 2020). These plants can thrive in warm, and humid environments. Rosemary is a shrub with fragrant evergreen needle-like leaves the stems are quadrangular, and erect in shape (de Macedo *et al*., 2020). Under natural conditions, *Rosmarinus* can attain height approximately 1 m to 2.5 m. In Europe *Rosmarinus* spp. due to having carnosic acid, has a very old reputation for improving memory, and has been used as a symbol for remembrance, as it shields the brain from the free radicals. *Rosmarinus* plant extracts have been used as traditional medicine to reduce hypertension, anxiety, headache, bloat, migraine, anorexia, as a local analgesia in the treatment of muscular pains, rheumatic disease. *Rosmarinus* essential oil also is very good for inhibition of hair loss due to vasodilatation and improved circulation. It is also useable in hepatic/jaundice diseases, excessive fatigue, and general weakness. *Rosmarinus* contains secondary metabolites, high contents of phenolic compounds (diterpenoids and flavonoids) and volatile compounds (de Macedo *et al*., 2020). All rosemary essential oils encompassantioxidant, antimicrobial, anti-inflammatory, and antiplatelet properties, mainly due to the presence of biologically active monoterpenes such as 1,8-cineol, camphor, and α-pinene limonene, camphene, camphor, and myrcene and phenolic compounds such as carnosic acid, carnosol, and rosmarinic acid (Borges *et al*., 2018).This significant antioxidant activity also accounts for other therapeutic abilities, including its anticancer and anti-diabetic mechanisms. REO is also used in cosmetic products. Due to its antioxidant and antimicrobial activity, REO is capable to extend the shelf-life of food products and maintain their quality during storage. Therefore, it used as a bio preservative, to prevent oxidation and microbial contamination and inhibitory in decomposition of poly-unsaturated triacylglycerolsin food industry and could be useful for replacing or even decreasing synthetic antioxidants in foods (Nieto *et al*., 2018; Hussain *et al*., 2020). REO is used in the treatment of dyspepsia and milder forms of spasmodic gastrointestinal

disorders, circulatory anomalies, as a complementary in the treatment of muscular or articular pain, mild pain reliever and inflammations (Raskovic *et al*., 2014). Rosemary oil is used in natural pesticides to kill certain insect. Rosemary is used in cosmetic preparations as a fragrance and as a skin conditioner in safe and it preserves cosmetics from degradation (Raskovic *et al*., (2014). Also, chemical variation and genetic diversity of essential oil of rosemary have been reported in subject to bioclimatic and geographic location (Jalali-Heravi *et al*., 2011). Hence, the objective of the current study was to assess the phytochemical composition of REO from *Rosmarinus officinalis* with antioxidant contents, antimicrobial, anti-inflammatory activity, anti-diabetic potential, antifungal activity against fungal strains associated with aspergillosis and mucormycosis.

Materials and Methods

Extraction of REO

REO was selected for current study. The essential oil used in the current study was kindly supplied by Wommune, Bioryca healthcare Pvt Ltd, India.

GC-FID analysis of REO

The gas chromatography (GC-FID) study was conducted using a Chemtron 2045 gas chromatograph coupled with a flame ionisation detector, with some modifications by Amrita *et al*. (2023). An 80-100% mesh Chromosorb W (HP) column made of stainless steel and 10% OV-17 was employed. With a flow rate of 30 ml/min, nitrogen gas was utilised as the carrier gas. The injector and detector were kept at respective temperatures of 250 °C and 200 °C. An injection of 0.2 µl of sample was made. For the ramping conditions in the oven, a starting temperature of 64 °C was kept and increased to 220 °C at a rate of 3 °C/min. Bioactive compounds were identified by comparing the relative retention time with known standards (eucalyptol, citral, eugenol and geraniol, Sigma-Aldrich (St. Louis, MO, USA) or with data published in the literature.

UV-Vis, FT-IR and fluorescent analysis

Using a UV-VIS spectrophotometer (Labtronics) with a slot width of 2 nm and a 10 mm cell at room temperature in the region of 200 nm to 400 nm, the UV-VIS spectral analysis of REO was carried out. Prior to testing, the concentrated extracted essential oil is appropriately diluted with methanol. A spectrum acquisition was recorded. The different bioactive compounds found in REO were identified using the Fourier Transfer Infra-Red (FT-IR) Spectroscopy and their particular functional groups. An FT-IR spectrophotometer (produced by Perkin Elmer, USA) was used to record the IR spectra. REO was studied using a scale of 4000-400 cm^{-1}, and the resulting spectrum was documented and assessed. The fluorescence spectrum of REO was measured with a Perkin Elmer spectrophotometer (FL6500). The parameters used were: slit width 10 nm, emission start at 410 nm, end at 700 nm, scans speed of 240 nm/min and excitation wavelength 310 nm. At room temperature (25 °C to 30 °C), all experiments were conducted.

In-vitro antioxidant analysis

Various antioxidants content like: total phenolic, total flavonoid and total tannin and associated antioxidant activities such as DPPH free radical scavenging activity, ABTS radical scavenging activity, hydroxyl radical scavenging activity, iron reducing assay, iron chelating assay were performed using various antioxidant assays as described in Amrita *et al.* (2023) with minor modifications. All determinations were performed in triplicate.

Total phenolics

Different amounts of REO (10-50 µl) were taken, and the final volume was diluted to 3 ml. Then 0.5ml of a 20 v/v% Folin-Ciocalteu reagent at a ratio of 1:10 was added. At room temperature, the reaction mixture was incubated for 5 minutes. 2 ml of Na_2CO_3 (20% w/v) were added after incubation. The resulting reaction mixture was then allowed to sit for an hour in complete darkness. Instead of using REO as a blank, absorbance at 650 nm was measured in comparison to pure water. Gallic acid was employed as a positive control for the calibration curve. Results were calculated using the usual equation y=0.001×+0.105, R2=0.995, and reported as gallic acid equivalent (GAE)/g REO.

Total flavonoid content

Various amounts of REO (10-50 µl) were mixed with 1 ml of methanol and 4 ml of distilled water. After adding 0.3 ml of $NaNO_3$ (5%w/v) to the mixture, it was let to sit at room temperature for 5 minutes. 0.3 ml of $AICI_3$ (10% w/v) was then added to the resultant mixture, which was once more incubated at room temperature for 6 min. 2 ml of NaOH (1M) was added and using distilled water, the reaction mixture's ultimate amount of was increased to 10 ml. The resulting mixture was incubated for 15 min in the dark. At 510 m, absorbance was measured following incubation. Rutin (1 mg/ml) was used to create the calibration curve. The results were expressed using the standard curve equation y=0.002×+0.094; R2=0.991 as mg of equivalent of rutin (RuE)/g of REO.

Total condensed tannins

Briefly, 3.0 ml of vanillin solution (4% w/v) in methanol and 1.5 ml of conc. HCI were added to various amounts of REO (10-50 µl) and incubated for 15 min at room temperature. At 500 nm, the absorbance was measured using methanol as a reference. A calibration curve was created using ascorbic acid. The results were calculated using the standard curve equation y= 0.000I×+0.001 R2 = 1 and expressed as g of ascorbic acid equivalent (AAE)/g REO.

In-vitro antioxidant analysis

Since the presence of a sample's total antioxidant content cannot ever be guaranteed by a single antioxidant activity. As a result, multiple antioxidant tests were used to investigate the antioxidant content of REO. Small adjustments were made to the assays carried out in accordance with Amrita *et al*. (2023). All determinations were performed in triplicate.

DPPH free radical scavenging activity

The DPPH assay was used to assess REO's capacity to neutralise free radicals. Various amounts of REO (10–50 µl) were mixed with 3 ml of the DPPH Solution. The reaction mixture was incubated for one hour in the dark at room temperature (25 °C-30 °C). After incubation at 517 nm, absorbance was measured using a blank solution of 82% methanol. A control sample of 3 ml of DPPH solution was used. An industry standard for comparison was ascorbic acid. The formula used to compute the percentage of DPPH radical scavenging activity is as follows: DPPH radical scavenging activity (%) = [(A0 A1)/A0 100], where A0 is the absorbance of the control (the reaction mixture without the sample/positive control). A1 = Test sample/standard absorbance.

ABTS radical scavenging activity

Three (3) ml of the produced ABTS radicals were mixed with various concentrations of REO (10-50 µl). The reaction mixture was incubated for 6 min at room temperature. At 734 nm, the absorbance was measured. As a positive control, ascorbic acid was used. The following equation was used to determine the percentage of ABTS+ radical scavenging: ABTS radical scavenging activity (%) = [(A0A1)/A0100], where A0

= ABTS radical absorbance when the sample or positive control is absent. A1 = Test sample/standard absorbance.

Nitric (NO_2^-) oxide radical scavenging activity

Three (3) ml of sodium nitroprusside solution (SNP, 10 mM in 1X PBS) solution was added to various amounts of REO (10-50 l). After that, the reaction mixture was incubated for 150 min at 25 °C. After incubation, 0.5 ml of the aforementioned reaction mixture was mixed with Griess reagent. At 546 nm, the absorbance was recorded. As a control, SNP in 1X PBS (3 ml) was regarded. The standard/positive control used was ascorbic acid. The following formula was used to express the nitric oxide radical scavenging activity of REO as far as % scavenging action: %age Scavenging activity is equal to A0-A1/A0*100, whereby A0 is the SNP's PBS absorbance. A1 is the test sample's (REO) absorbance.

Hydroxyl radical scavenging activity

One (1) ml of phenanthroline (0.75 mM), 2 ml of sodium phosphate buffer (0.2 M), 1 ml of $FeSO_4$ (0.75 mM), and 1 ml of H_2O_2 (0.01%) made up the reaction mixture for the production of the hydroxyl radical. REO was progressively added (10–50 µl) to this reaction mixture, and the mixture was then incubated at 37 °C for 30 min. Following the incubation period, absorbance was measured at 532 nm using deionized water as the blank solution and ascorbic acid as the reference substance. The following formula was used to compute the hydroxyl radical scavenging activity: Scavenging Activity (%) = (Abs of sample- Abs of blank)/ (Abs of control-Abs of blank) *100. In contrast, Abs of control is the amount of water that absorbs in the reaction mixture in lieu of the sample (REO) and H_2O_2.

Iron reducing assay

Potassium Ferricyanide (1% w/v) and sodium phosphate buffer (200 mM; 6.6 pH) make up the reaction mixture in 1.25 ml. TTEO was then added in increasing amount (10–50 µl), and the mixture was then incubated for 20 min at 50 °C. After adding 2.5 ml of TCA (10% v/v/), the mixture was centrifuged at 3000 rpm for 10 minutes. 100 µl of $FeCl_2$ and 1.5 ml of distilled water were added to 1.5 ml of the top layer. After 10 to 15 minutes, an O.D. at 700 nm was seen using distilled water as the reference solution. Ascorbic acid served as a positive control. Increased absorbance corresponds to increased lowering capacity.

Iron chelating assay

In this experiment, various volumes of REO (10-50 µl) were combined with 900 µl of $FeSO_4$ (500 M), and then 78 µl of 1,10-phenanthroline (0.25% v/v) were added. The resultant solution's absorbance was measured at 510 nm using water as a reference. EDTA was considered the positive control. REO's capacity to chelate Fe^{2+} was determined using the formula below. Cheating capacity (%) is calculated as (ACONTROL - ASAMPLE)/ACONTROL 100. Where ACONTROL stands for the absorbance of $FeSO_4$ and ASAMPLE is the reaction mixture's absorbance when either the sample (REO) or the positive control (EDTA) are present.

Anti-inflammatory activity (protein denaturation) of REO

Protein Denaturant Assay was also carried out and researched utilising Fluorescent Assay (Agnish *et al*., 2022). The following ingredients were added to the reaction mixture in the following order: 400 µl BSA (1% w/v), 4.78 ml PBS (1X; pH 6.4), and REO (10-50 µl). The resulting combination was then heated for 5 min at 70 °C after 15 minutes of incubation at 37 °C in a water bath. The reaction mixture was cooled right away. Using a Perkin Elmer Fluorescence Spectrophotometer FL6500, fluorescence analysis was performed on the reaction mixture (1 ml) in quartz cuvettes. The emission spectrum varied from 300 to 400 nm, while the excitation wavelength was 280 m. The tests were all carried out at 30 °C, or room temperature.

α-Amylase inhibition assay of REO

Test tubes containing various aliquots of REO (50–250 µl) were filled with 125 µl of a-amylase solution (5 mg/ml) and 0.5 ml of sodium phosphate buffer (0.02M, 6.9 pH). The solution was pre-incubated for 10 min at 25 °C. Then 500 µl of a 2% (w/v) starch solution made in 0.03 M sodium phosphate (pH: 6.9) was added, and the mixture was incubated at 25 °C for an additional 10 min. The reaction was stopped by adding 0.5 ml of DNS reagent. The test tubes were once more placed in a boiling water bath for five minutes before being cooled to room temperature. The standard/positive control was administered as acarbose (10 mg/ml). The samples were diluted with 6ml of distilled water after incubation in order to test O.D. at 540 m. The following formula was used to determine the activity of the -amylase inhibitor: ACONTROL-ASAMPLE/ACONTROL*100 equals %age Inhibition. Where, ACONTROL =Absorbance of reaction mixture without sample/positive control. ASAMPLE = Absorbance in the presence of sample/positive control.

Mode of α-amylase inhibition

The mode of REO's -amylase inhibition was carried out as follows. In one set of tubes, 75 µl of REO was pre-incubated for 10 min at 25 °C with 200 µl of -amylase solution and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9). Another set of tubes contained 500 µl of 0.02 M sodium phosphate buffer (pH 6.9), which was pre-incubated with -amylase. The reaction was started by adding varying amounts of starch solution at increasing concentrations of (2-8 mg/ml) to both sets of tubes. The reaction was then stopped by adding 500 l of the dinitrosalicyclic acid (DNS) reagent after the mixture had been incubated at 25 °C for 10 min. The amount of released reducing sugars and their concentrations were calculated using the maltose standard curve. A double reciprocal plot (1/V versus 1/S) between substrate concentration (S) and velocity (V) was plotted. By analysing Lineweaver-burk plot using Michaelis-Menten Kinetics mode of inhibition of REO on α-amylase activity was recognized.

In-vitro anti-bacterial activity of REO

The anti-bacterial activity of REO was assessed using the agar disc diffusion method against four test species, including the gram-positive *Bacillus subtilis* (MTCC 121), gram-negative *Escherichia coli* (MTCC 40), and gram-positive *Staphylococcus aureus* (MTCC 3160). The Chandigarh-based IMTECH Institute of Microbial Technology was where the microorganisms were bought. All strains' inoculums were made from 12-hour-old cultures, and 0.5 MFU of the McFarland standard was utilised as a reference to modify the turbidity of the bacterial suspension culture. 1.5 x 10^8 cells/ml was the rough cell density. Paper discs which were flooded with 50-200 µl of REO were placed in the centre of the Petri plates. The plates were left at room temperature for 20 minutes to allow oil diffusion, followed by incubation at 37 °C for 24 hrs. After incubation, the antimicrobial activity was observed as a zone of inhibition around the discs. The positive control was streptomycin (10 mg/ml). The zone of inhibition (the radius created around the disc) was noted down after the incubation plates were visualised under a transilluminator, indicating REO's antibacterial activity.

Determination of anti-fungal activity of REO

Following the procedure outlined by Amrita *et al*. (2023), the food Poisoning technique was also employed to assess the antifungal activity of REO. In this procedure, petri plates were prepared with various amounts of REO (5-200 µl/ml) and 25 ml of sterile potato dextrose agar substrate. A sterilised cork borer was used to remove mycelial agar discs (10 mm in diameter) from the actively budding zone of a 7-day-old pure culture of *Aspergillus flavus* (MT9CC 277), *Aspergillus fumigatum* (MTCC 343), and *Mucor* spp. (MTCC 3373). The fungus discs in the centre of the petri plates served as aseptic inoculants. For each fungal strain, blank or negative control plates were also created, however they contain water instead of REO. As a positive control, ketoconazole (25 µg/ml) was administered. Each plate underwent incubation. The findings of 15 days

of observation on the plates were recorded. By using the formula, the antifungal activity of REO against all fungal strains was expressed as a % suppression of mycelial growth. Mycelial growth inhibition = C-T/C*100, Where C and T are the mean mycelial diameter of negative control and treated sample (REO).

Membrane integrity assay

Following the procedure outlined by (Amrita *et al*., 2023), the impact of RNE on membrane integrity was investigated against the bacterial strains *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 40), *Staphylococcus aureus* (MTCC 3160), and *Bacillus subtilis* (MTCC 121). The bacteria were treated with 200 µl of pure RNE (A_1) at a concentration of 1×10^8 CFU/mL. As a positive control (A_0), bacterial cells were treated with 200 µl of Triton X-100. Cells that had not been treated served as a negative control. The treated cells were observed for 12 hours after being incubated in an orbital shaker incubator (REMI instruments) at 37 °C and 150 rpm. After an equal amount of time had passed, 1 ml of each bacterial strain's sample was taken from each flask. After that, the cells were centrifuged for 10 minutes at 6000 rpm in order to extract the cytoplasmic contents. With the use of a single beam UV-Spectrophotometer from Labtronics, the resulting supernatant was collected and measured at 260 nm. The amount of UV-absorbing materials that seeped out of the bacterial cells was expressed as the "cytoplasmic content released" (CCR), expressed in percentage (%) units. The following formula was used to compute the proportion of cytoplasmic content released: CCR (%) = A_1/A_0x 100.

Statistical analysis

Tukey HSD test by SPSS 26.0 was used to determine p (< 0.05) values (https://www.socscistatistics.com/tests). All experiments and analysis were performed in triplicate manner (n = 3). IC_{50} values of REO providing 50% inhibition was calculated at https://www.aatbio.com/tools/ic50-calculator.

Results and Discussion

Essential oils are one of several secondary metabolites that are known to be biologically active and have strong pharmacological properties, because they are safer than synthetic ones (Agnish *et al*., 2022). In the current study the total phenolics, total flavonoids, and total condensed tannins were estimated to be 0.53 mg/g GAE, 0.82 mg/g RE, and 32.3 mg/g AE respectively. The aforementioned phytocompounds are plant secondary metabolites which have antioxidant activity *in vitro* and *in vivo* conditions. Earlier studies have documented those plants poses medicinal and redox properties due to having these chemical structures (Khan *et al*., 2012). Previous studies have demonstrated that biological factors like developmental stage, genotype, organ and ontogeny, as well as edaphic and harsh climatic environmental factors like high solar light, hot temperature, water stress, salinity, etc., have a significant impact on the amount of secondary metabolites and their antioxidant activities (Medini *et al*., 2014). Plants respond to oxidative stress and photodamage with the production of polyphenolics through secondary metabolic pathways (Yasin *et al*., 2021). The data of present study revealed that REO contained notable amount of polyphenolics compounds endowed with high antioxidant. We, therefore opine that polyphenolics may contribute to the medicinal value of *Rosmarinus officinalis*. These findings provided good pharmacological logic for *Rosmarinus officinalis* use in folk and traditional medicine. The presence of high content of polyphenolics indicated the effectiveness of REO as potent source of bioactive compounds from *Rosmarinus officinalis*.This study data provided clue that due to its richness in bioactive components, essential oils can reduce the risk of many diseases by scavenging free radicals in biological pathways (Agnish *et al*., 2022).It has been reported in previous investigations that bioactive

compounds such as phenolics from medicinal plants are immensely used to mitigate many diseases and scavenging activities (Khan *et al*., 2012).

Gas chromatography analysis

The chromatogram obtained by GC-FID was depicted in Table 1, the observed peaks and their retention time is also given in the Figure 1. The GC-FID analysis of REO extracted from *Rosmarinus officinalis* resulted in the identification of 37 compounds accounting for total 100%. REO is endowed with major components like:cis-3-Hexanol (18%), Vertocitral C (16%), Camphene (9%), α-Pinene (8%), Humulene oxide (7.6%), 1,8-Cineole (4.1%), Cumenol (4%), β-Caryophyllene (3.8%), and Limonene (3.2%). REO is characterized by the high amount of cis-3-Hexanol, Vertocitral C, Camphene, α-Pinene, Humulene oxide, 1,8-Cineole, Cumenol, β-Caryophyllene and Limonene similar to those found in some other reports (Graber *et al*., 2010; Jalali-Heravi *et al*., 2022). REO is also endowed with many other minor bioactive constituents. The small peaks may be credited to the bioactive compounds present in the minor concentrations. The composition of the essential oil observed was found to be different from the previous reports. REO composition is affected by various factors, such as different environmental factors (climate, seasonal or geographical), weather, soil humidity, extraction method, distance between plants, harvest time, season of harvesting and drying method, or storage conditions and their genotype (Borotová *et al*., 2022). Some earlier studies also documented the presence of major and minor constituents but with varied composition. For instance, in our study, camphene was the dominant component with 9%, however, Graber *et al.* (2010) reported Camphene composition with 7%. Similarly, Jalali-Heravi *et al*. (2022) also reported camphene in REO with 0.3%. Authors also documented less amount of β-Pinene with 0.1% which was less than our REO (1.8%). A study by Rahman *et al.* (2007) reported cis-3-Hexanol with 0.08% where as in our REO it was predominant component with 18%. Same authors observed Camphene 5.8% than ours REO that was endowed with 9%. The concentration of Terpinen-4-ol was 3.2% in our REO than 1.4% reported by Rahman *et al.* (2007). Due to richness of these bioactives, now a days, REO is expanding fame in various trades and being used in aromatherapy, cosmetics, food, herbal and allopathic medicines, and as natural fungicide, biocide, and preservative.

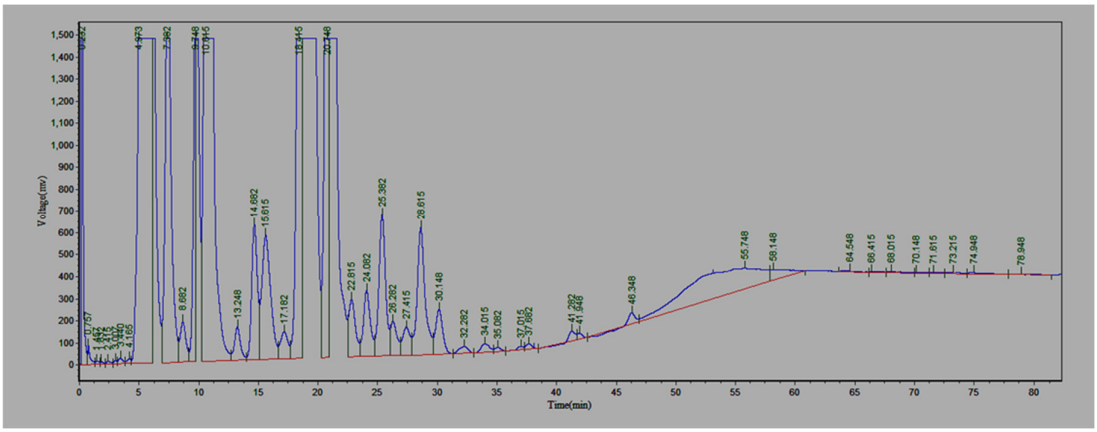


Figure 1. GC-FID profile of REO, arrow indicates major bioactive compound

Table. GC-FID components in REO

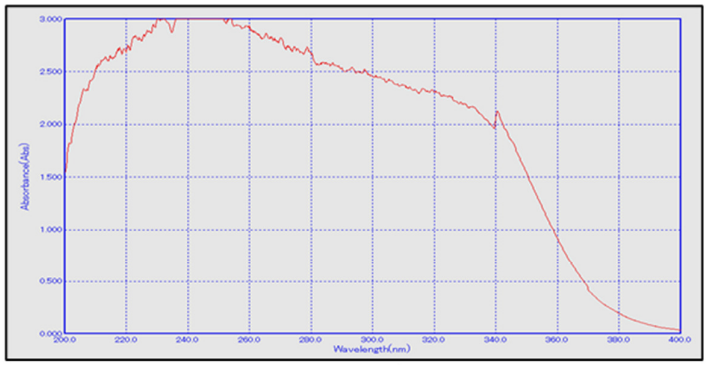
Peak no.	Retention time (min)	Bioactive compound	Conc.
1	0.232	-	2.4494
2	0.757	-	0.1904
3	1.457	-	0.0465
4	1.832	-	0.0376
5	2.415	Methyl butanoate	0.0473
6	3.007	Cyclohexene	0.0395
7	3.440	Thiazole	0.1045
8	4.165	Hexenol «2E)->	0.0704
9	4.973	cis-3-Hexanol	18.4082
10	7.382	Camphene	9.1037
11	8.682	α-Terpinene	0.9471
12	9.748	Limonene	3.2614
13	10.615	Vertocitral C <cis>	16.2627
14	13.248	Camphor	0.8144
15	14.682	Terpinen-4-ol	3.2309
16	15.615	Cumenol<o>	4.4644
17	17.182	Thymol, methyl ether	0.8167
18	18.415	α-Pinene	8.3285
19	20.748	Cyclohexanol acetate <trans-2-tert-butyl>	6.6781
20	22.815	α-Phellandrene	1.5835
21	24.082	β-Pinene	1.8518
22	25.382	β-Caryophyllene	3.8781
23	26.282	α-Thunjene	0.9247
24	27.415	p-Cymene	0.8704
25	28.615	1,8-Cineole	4.1131
26	30.148	α-Terpinolene	1.3053
27	32.282	Exo-Fenchol	0.2590
28	34.015	Cis-dihydrocarvone	0.2995
29	35.082	Methyl bornyl ether	0.1056
30	37.015	Homo myrtenol	0.0806
31	37.682	Bornyl acetate	0.1381
32	41.282	Khusinol acetate	0.2159
33	41.948	Isotorquatone	0.1256
34	46.348	Isoprenylcinnamate «Z)-methyl->	0.1723
35	55.748	Humulene oxide	7.6802
36	58.148	Manool	0.7299
37	64.548	Hexacosane	0.0260
38	66.415	-	0.0303
39	68.015	-	0.0609
40	70.148	-	0.0330
41	71.615	Nonacosane	0.0403
42	73.215	-	0.0557
43	74.948	Triacontane	0.0835
44	78.948	Dotriacontane	0.0351

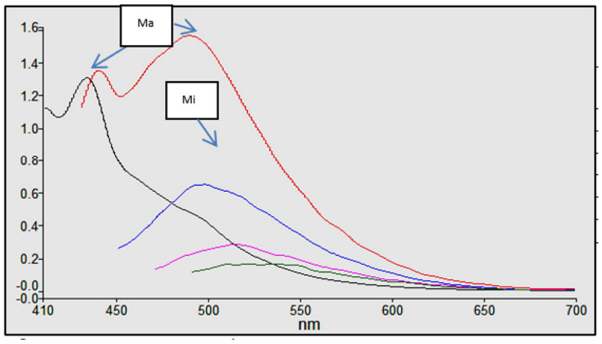
Fingerprint analysis

Spectroscopy offers a very comprehensive understanding of the qualitative and quantitative compositions of herbal derivatives and bioactive compounds of plant origin. In this context, UV-VIS, fluorescence, and FT-IR spectroscopy techniques can be used jointly or separately as traditional approaches for detecting phytocomponents (Agnish *et al*., 2022). The qualitative UV Spectroscopy profile of REO revealed different sharp peaks from 240-360 nm with absorbance of 2.0-3.0 (Figure 2), signifying presence of secondary metabolites such as flavonoids, tannins and phenolics (Agnish *et al*., 2022). Spectroscopic tests displaying the distinctive spectra acquired in the ultraviolet and visible spectrum proved the existence of phytochemicals. An earlier work (Rani et al., 2016) that reported the UV-Vis spectra of leaf extract in the absorption region between 270 and 340 nm verified similar findings in *Meizotropis pellita*. Dhivya and Kalaichelvi (2017) also reported flavonoid spectra mainly consist of two absorption maxima in the ranges of 230-285 nm and 300-350 nm in *Sarcostemma brevistigma*. A study by Donkor et al (2019) displayed absorption maxima in the area of 203.0-385.1 nm, 202-423.0 nm, and 203.0-379.0 nm in the UV-Vis spectra of the extracts of the different organs like leaves, unripe fruits, and mature fruit, verifying the presence of phytochemicals.

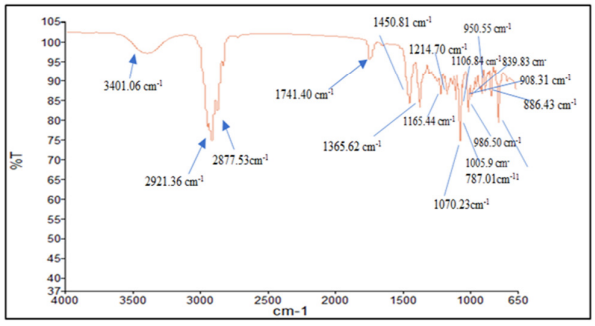
Another strategy for the accurate and practical detection of plant bioactives, fluorescent spectroscopy is preferred. The fluorescent spectrum of REO is given in Figure 2B. They are grouped together on the basis of excitation wavelengths. In almost all spectra, notable two peaks were detected, one minor in the Red Florescent Region (RFR) at 500 nm and major in the Green Fluorescent Region (GFR) at 420 nm. It has been proposed that polyphenolic compounds such as flavonoids, flavins, and terpenoids are the bioactives in the 500 nm range (GFR) (Mylle *et al*., 2013). As shown by a small peak, other bioactive compounds were also found in the Red-Florescent Region (RFR). These additional compounds may have accumulated due to the presence of other fluorescent chemicals such anthocyanins, phenolics, alkaloids, and aromatic benzenoids. Further, peaks of different intensities were detected as per their excitation wavelength. As excitation wavelength increased from ƛ390 nm to ƛ470 nm, a drastic change in peak intensity was also noticed. For instance, maximum intensity of 1.4 units was exhibited at ƛ390 nm, which was reduced to 0.2 units at ƛ470 nm. Differences in the fluorescence intensities in fluorescent spectra may be related to chemical composition of REO.

The Fourier Transform Infrared Spectrophotometer (FT-IR) is the most potent, quick, and non-destructive approach for identifying plant powders or extracts, as well as for identifying the various bonds and functional groups that may be present in the extracts (Chen *et al*., 2016). The absorption bands in the IR spectrum area 3000-600 cm^{-1}, or the "fingerprint", are characterised by spectral vibrations of the molecular structures, such as deformation, combining, and harmonic bands. FT-IR peak values for REO are given in Figure 2C. In FT-IR spectrum peak at 3401 cm^{-1} indicated O–H stretching due to hydroxyl group. Peaks at 2921.36, 2877.53, 1450.81, 1005.95 indicated C-H stretching due to alkanes, peak at 1741.40 due to aldehydes, 1214.70 due to vinyl ether, 1106.84 depicted C=O bonding due to carbohydrates indicates, and peak at 1365.62 indicated C-O bonding due to esters. Peak at 1070.23 indicated S-O stretching due to sulphoxide, and peaks at 1165.44, 986.50, 950.55, 886.43, 839.83 and 787.01 indicated C=C bonding due to alkenes. Donkor *et al*. (2019) also reported the presence of secondary metabolites in *Duranta erecta* L. like: Alcohols, phenols, alkanes, aldehydes, ketones, aromatics, aliphatic amines, aromatic amines, amides, carboxylic acids, esters, nitro compounds, alkynes, primary and secondary amines, and alkyl halides were all detected by FT-IR analysis. All these observations imply that all REO is rich in secondary metabolites. All these compounds belong to the secondary plant metabolites (Paulraj*et al*., 2011). All these observations imply that all REO is rich in secondary metabolites. The presence of above said secondary metabolites may be the reason for medicinal properties of tea tree oil.





	Peak intensity (INT) x 10^4		Fluorescence wavelength	
	Ma	Mi	Ma	Mi
— 390	1.4	0.5	420	500
— 410	1.5	1.3	500	450
— 430	-	0.6	-	550
— 450	-	0.3	-	550
— 470	-	0.2	-	550



Peak No.	X (CM^{-1})	Vibration mode	Functional group
1.	3401.06	N-H Stretching	Aliphatic 1° Amine
2.	2921.36	C-H asymmetric stretching	Alkane
3.	2877.53	C-H Stretching	Alkane
4.	1741.40	C=O stretching	Aldehyde
5.	1450.81	C-H bonding	Alkane
6.	1365.62	C-O stretching	Ester
7.	1214.70	C=O bonding	Vinyl ether
8.	1165.44	C=C Stretching	Alkene
9.	1106.84	C=O bonding	Carbohydrates
10.	1070.23	S-O stretching	Sulfoxide
11.	1005.95	C-H bonding	Alkane
12.	986.50	C=C bonding	Alkene
13.	950.55	C=C Bonding	Alkene
14.	908.31	C=C bonding	Alkene
15.	886.43	C=C bonding	Alkene
16	839.83	C=C bonding	Alkene
17.	787.01	C=C bonding	Alkene

Figure 2. UV (A), Fluorescent (B) and FT-IR (C) spectra of REO
Ma: major, Mi: minor

In-vitro antioxidant analysis of REO

The estimation of a medicinal plant's therapeutic potential typically involves assessing its antioxidant potential and related activities. Borrás-Linares*et al* (2014) reported that due to richness of numerous bioactive molecules, essential oil from different *Rosmarinus* spp. have potentially strong antioxidant properties. Earlier reports reported that molecules such as phenolics, flavonoids, and tannins, etc., were accountable for the therapeutic value of the plants in which they were found due to their antioxidant activity (de Macedo *et al*., 2020). Authors stated that the aromatic plants based natural antioxidants from are fetching progressively importance, not only in the nutritional area (food preservation and stability) but also in preventive and complementary medicine. This study data revealed that REO endowed with powerful antioxidant activities. The IC_{50} value of REO is given in Table 2. Performance wises the antioxidant potential of REO was observed in the following order: OH^- (16.6118), iron chelating (32.0115), iron reducing (92.534), DPPH (101.929), and ABTS (172.2765). The linear correlation analysis was also followed to check correlation between various antioxidant activities and total flavonoid content, total tannin content, total phenolic content, results are given in Table 3.

Table 2. IC_{50} values of different antioxidant assays of REO

Activity	IC_{50} Value [µl/ml]
DPPH scavenging activity	101.929
ABTS activity	172.2765
Hydroxyl radical scavenging activity	16.6118
Iron chelating activity	32.0115
Iron reducing power	92.534

Table 3. R^2 Values of different activities of REO

Activity	DPPH activity	ABTS scavenging activity	Iron reducing activity	Iron chelating activity	Hydroxyl radical scavenging activity
Total flavonoid content	0.8354	0.8659	0.9108	0.7726	0.820
Total phenolic content	0.7373	0.7692	0.8913	0.8436	0.8250
Total condensed tannins	0.8973	0.8775	0.9019	0.993	0.8841

A strong correlation between polyphenolics content and different antioxidant activities was observed. For instance, R^2 value for Flavonoid content and iron reducing activity was 0.91, and R^2 value for total phenolic content and iron reducing activity was 0.89. R^2 value for total condensed tannins content and hydroxyl radical scavenging activity and iron chelating activity was 0.88 and 0.99, respectively. This observation was in consonance with earlier studies reporting tight correlation with polyphenolics contents and antioxidant activities (Khan *et al*., 2012). A positive correlation between the amount of phenolic compounds and the antioxidant activity of plants has been reported in numerous studies (Brasileiro *et al*. 2015). Due to this antioxidant and antimicrobial activities REO from various plant species are used as preservative in food industry (Nieto *et al*., 2018). Herbal products may contain a variety of substances with various antioxidant capabilities. The antioxidant values of herbal derivatives are connected to one another (Khan *et al*., 2012). We therefore believe that the presence of flavonoids and other phenolic compounds found in this study may be the cause of the essential oil's notable antioxidant effects. Any bioactive compound's abundance in antioxidant

properties is a sign of its potential for usage as a food or medicine supplement to prevent the oxidative damage that free radicals cause to biomolecules, which in turn revitalises bodily functioning (Khan *et al*., 2012).

Due to the complex antioxidant and phytochemical nature of essential oils, previous studies have shown that no single assay can accurately determine the antioxidant assay for an essential oil. Therefore, multiple methods must be used to evaluate the total antioxidant activities, taking into account each possible mechanism of the antioxidant potential of an essential oil's bioactive constituents. A number of *in-vitro* assays like ABTS, DPPH, OH^- scavenging, iron reducing, and iron chelation assay were evaluated to measure antioxidant potential of REO.

In the present study REO at different volumes was used to measure DPPH radical scavenging activity, as shown in Figure 3. It is clear from the results, that the REO exhibited tremendous DPPH radical scavenging activity as 94% activity at its higher concentration 50 µl. Further, REO showed dose dependent increase in the ABTS scavenging activity. For instance: at 50 µl which was the highest volume of REO tested showed 13% scavenging activity. Whereas 10 µl of REO used, only 2.7% activity was noticed. REO's capacity to scavenge OH^- radicals was also observed. The results are shown in Figure 3. It was apparent that REO showed dose dependent hydroxyl radical scavenging activity, with 63% activity at its highest test volume of 50 µl. Iron reducing assay of REO was also determined in the present study and results are shown in Figure 4.

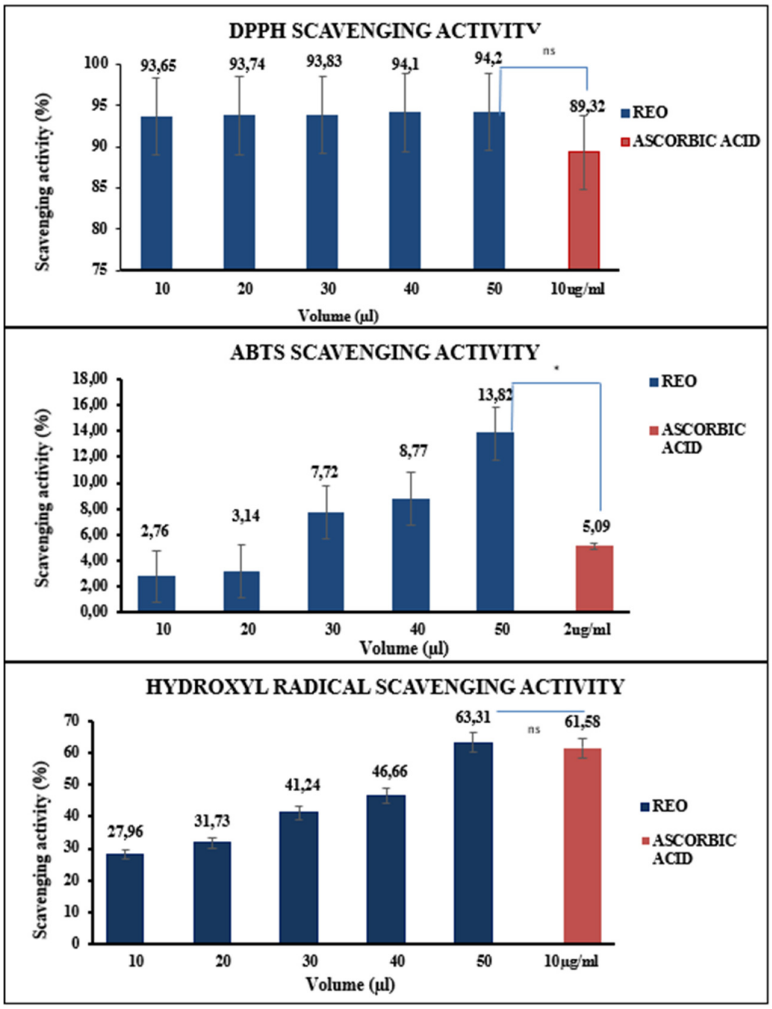


Figure 3. Various antioxidant activities of REO

* indicates significant difference at P≤0.05 vs positive control

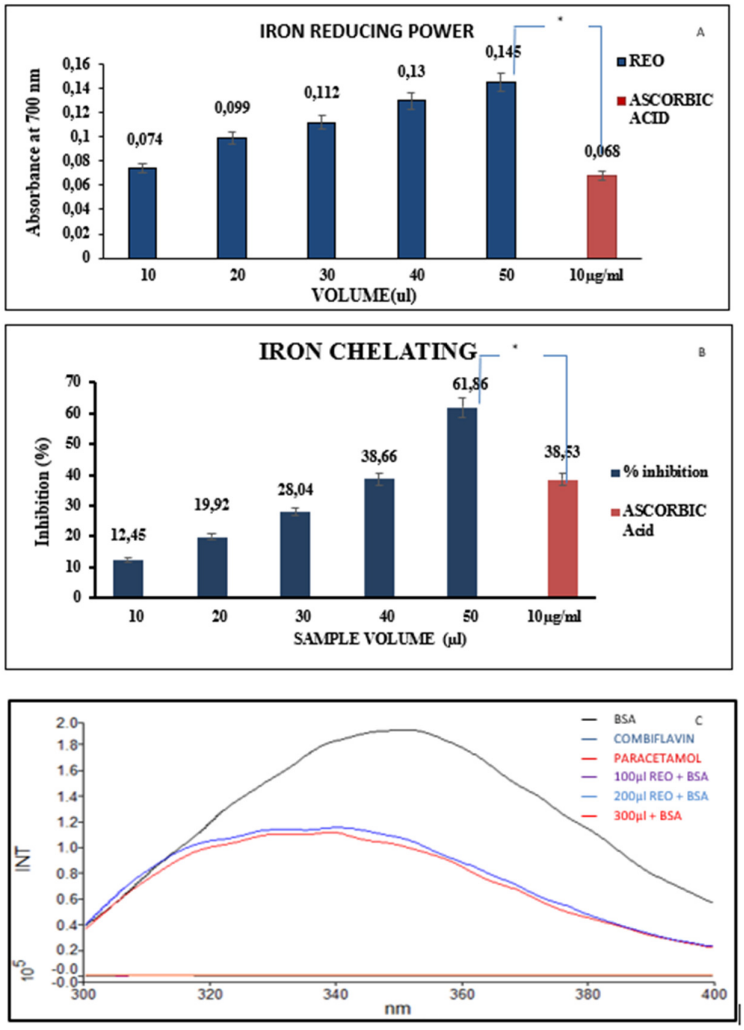


Figure 4. Various antioxidant activities (A), (B) and anti- inflammatory (Protein Denaturation) activity (C) of REO

* indicates significant difference at P≤0.05 vs positive control

At different REO fractions, reduction potential was taken. At 50µ l, the REO showed 0.14 absorbance values. A comparison was made with reference compound ascorbic acid (Vitamin C), well-known potent antioxidant and free radical scavenger, which exhibited 0.06 absorbance value. Statistical test performed showed significantly (P≤0.05) higher reducing ability of REO than positive control. The reducing ability is amplified with increased absorbance. This was in line with earlier studies (Joshi *et al*., 2010) citing role of essential oil as antioxidants. The iron chelating activity of REO was determined in the current study by estimating the metal chelating tendencies of REO. Figure 4 showed chelating activity of REO at different fractions. A commercially used metal chelating agent EDTA was used as reference compound. From results obtained it was clear that REO showed higher metal chelating activity 61% at its higher volume at 50 µl as compared to EDTA which showed 38% activity at concentration of 10 µg/ml. Over all, these studies come to the conclusion that REO antioxidant effects may be attributed to the presence of polyphenolics and major and minor bioactives such a cis-3-Hexanol, vertocitral C, Camphene, α-Pinene, Humulene oxide, 1,8-Cineole, Cumenol, β-Caryophyllen and Limonene bioactive components *in-toto*. According to Khan *et al.* (2012), antioxidant activities against DPPS and ABTS were related to concentration, chemical structures and

polymerization degree of antioxidants. It was also reported that high molecular weight phenolics like tannins have more ability to mitigate free radicals. It was also reported that the α-pinene a compound present in our REO with a percentage of 8.3% was found to be endowed with a strong antiradical and ferric reducing power activity (Wang *et al*. 2008). Finally, 1,8-cineole, β-pinene compound present in our REO with a percentage of 4.1% and 1.8%, respectively, was reported to have strong antioxidant activity. Authors from this study also stated that in addition to the major compounds, minor components may also contribute significantly to the activity of REO. Authors noticed that antioxidant activity is variable and related to chemical composition and can be affected by specific substances. Previously, Viuda-Martos *et al.* (2010) also documented that oxygenated monoterpene, probably monoterpenoid ketones may have the greatest contribution to the antioxidant capacity of REO. A study postulated that this natural antioxidant has superior properties than synthetic butylatedhydroxytoluene, which gives the likelihood to avert food oxidation (Rašković *et al*., 2014). Some other authors also described that the antioxidant activity of REO was related to the presence of compounds, such as verbenone, borneol alcoholic ethers and phenolic compounds, 1,8-cineol, α-pinene, β-pinene, α-thujene, trans-caryophyllene, β-thujone, borneol, and camphor (Beretta *et al*., 2011; Kadri *et al*., 2011; Ojeda Sana *et al*., 2013). Authors pointed out that as compared to other natural antioxidants like vitamin C, vitamin E, and quercetin, REO oil exhibited strong free radical scavenging properties which was due to the presence of phenols in this oil (Gabriela *et al*., 2018).

Anti-inflammatory (protein denaturation) activity

Inflammation is a common defensive response that are brought on by tissue damage or disease, and it has the power to fight off invaders (microorganisms and non-self-cells) as well as destroy dead or damaged host cells (Miguel *et al*., 2010). Protein denaturation is the primary cause of tissue inflammation, which causes loss of function, swelling, redness, and heat release in the vicinity of the affected areas. It was proposed that bioactives with the ability to prevent heat-induced protein denaturation could be employed as effective anti-inflammatory drugs (Agnish *et al*., 2022). In the present study, the fluorescence method was used to investigate the protein denaturation inhibitory effect of REO. Characteristic fluorescent spectra of denatured BSA (Bovine Serum Albumin) and BSA + REO at different concentrations are shown in Figure 4C. REO demonstrated a remarkable improvement in the suppression of protein denaturation, as seen by the significant drop in fluorescence intensity i.e. 1.8×10^5 to 0.0 that occurs following the addition of REO to BSA. These findings provide strong evidence for the REO's potential as an anti-inflammatory agent. The high protein denaturation inhibition ability of REO might be credited to complex compounds having synergistic associations of numerous compounds or involvement of minor components. Phenolics compounds like tannins, phenolics and flavonoids possess diverse biological activities such as anti-inflammatory (Khan *et al*., 2021). In addition to acting as an anti-inflammatory mediator due to its antioxidant activity, REO may also effectively protect the body by limiting the growth of inflammatory cells without impairing those cells' ability to generate anti-inflammatory cytokines (Caldefie-Chézet *et al*., 2006).

Anti-diabetic (α-amylase inhibition) activity

The goal of alpha-amylase inhibition is to slow down the metabolism of carbohydrates using a variety of inhibitors to regulate blood sugar levels (McCue *et al*., 2005). α-Amylase Inhibition potential of REO was determined as shown in Figure 6. It is clear from the Figure 6 that REO was found to be a very potent inhibitor of α-Amylase in dose -dependent manner. At a higher amount of 250 µl, REO had an inhibition percentage of 26.29% as compared with acarbose (a known inhibitor for α-amylase), which showed 4.55% inhibition at 10 µg/ml concentration. The statistical analysis performed showed the existence of highly significant difference between the α-Amylase Inhibition of the acarbose used as positive control and the α-Amylase Inhibition of the REO ($p < 0.05$). One of the methods used to treat diabetes mellitus is the inhibition of enzymes that break down carbohydrates, such as α-amylase. This lowers the body's blood sugar levels by reducing gastrointestinal

glucose absorption (McCue *et al*., 2005). The mode of inhibition of REO was determined by Lineweaver-burk plot and Michaelis-Menten likewise showed Figure 5, that displays REO inhibits α-amylase competitively. The proposal states that the REO's active components compete with the enzyme's substrate for binding to the active site, preventing the breakdown of oligosaccharides into disaccharides. Literature research demonstrated that phytochemicals like flavonoids, which have the potential to inhibit a-amylase activity, account for the majority of the inhibitory effect of plant extracts (Kazeem *et al*., 2013). Sahin *et al*. (2010) mentioned that the p-cymene, and α-pinene two monoterpenes endowed with an inhibitory activity of α-amylase. The presence of several phytochemicals like flavonoids, saponins, and tannins in the REO may be the cause of its inhibitory effects. Previous research on α-amylase inhibitors extracted from therapeutic plants suggests that a number of these inhibitors may belong to the flavonoid class, which possesses characteristics that block α-amylase activity (Kwon *et al*., 2007).

Antibacterial activity

Essential oil extracted from *Rosmarinus officinalis* was assessed quantitatively for determination of *in-vitro* antibacterial activity against Gram-negative *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 424) and Gram-positive *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 121) by agar disc diffusion method. The current study revealed that REO exhibited strong antibacterial activity as shown in Figure 6 and Table 4. REO showed strong antibacterial activity against *Bacillus subtilis* (MTCC 121 G^+) with inhibition zone of 2.5 cm. REO exhibited moderate activity against tested pathogens *P. aeruginosa* (MTCC 424 G^-) with inhibition zone in the range of 1.7 cm. The presence of many targets or a single target may be the cause of the variation in REO's antibacterial efficacy against tested pathogens. The antimicrobial activity of REO may ascribe due to chemical composition and the presence of major and minor bioactive components that affect hydrolytic enzyme inhibition (proteases) or inhibited partners such as: microbial adhesions and cell wall envelop proteins (Su *et al*., 2006). Furthermore, the multi-component nature of essential oils enables them to have multiple target sites for their antimicrobial action, hence, they may be able to deal with otherwise resistant microorganisms. According to Lee *et al*. (2013), the antibacterial action of essential oils and their respective constituents is linked to their lipophilic nature which enables them to accumulate in membranes, thus making cell membranes the main target site. However, the antibacterial action of essential oils has also been attributed to bacterial cell wall degradation, reduction in proton motive force and reduction of intracellular adenosine triphosphate (ATP) levels. Previous studies also cited that antimicrobial activity is also variable and dependent on factors such as chemical composition (Lee *et al*., 2013). Earlier studies have cited that anti-microbial activity was not always related to the high content of one major chemical compound, rather than to synergic effects between major and minor components (Elaissi *et al*., 2012). Cordeiro *et al*. (2020) reported terpinen-4-ol as a major antimicrobial contributor in REO. However, a study by Lee *et al*. (2013) also made a mention that minor components also contribute to the antimicrobial efficacy of essential oils. Swamy *et al*. (2016) also reported 1,8 cineole, component detected in our REO, has been reported to have antibacterial activity. In another study by Siramon *et al*. (2007), polyphenolics have been reported behind the antimicrobial activity. Authors claimed that these polyphenolics have tendency to cross the cell membranes and thereby disrupt biological reactions, proton motive force, active transport and coagulation of the cellular contents. Interestingly, our study REO exhibited high antimicrobial toxicity toward G^- bacteria which was a noteworthy observation as earlier studies claimed that the G^- bacteria are more resistant than G^+ bacteria due to presence of thick cell wall that allow G^- bacteria to express resistant to mostly antibiotics and toxic drugs (Swamy *et al*., (2016).

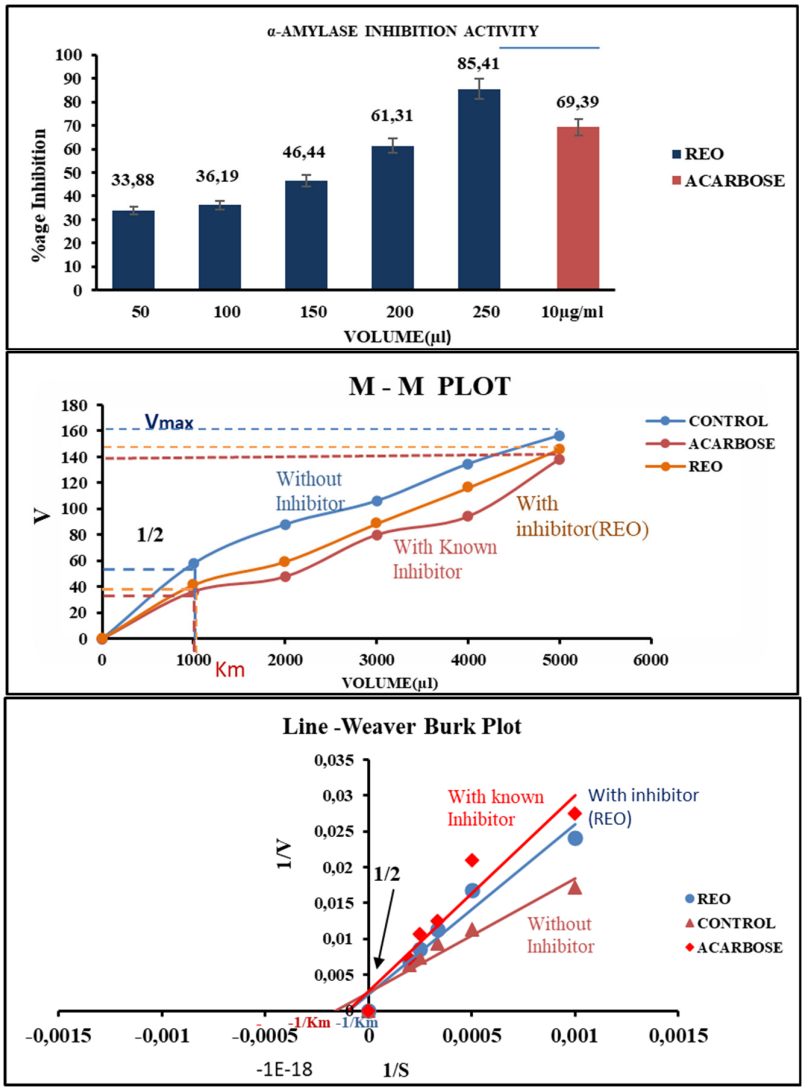


Figure 5. α-Amylase inhibition activity, MM plot and Lineweaver-Burk plot showing competitive inhibition of α- amylase by REO

* indicates significant difference at P≤0.05 vs positive control

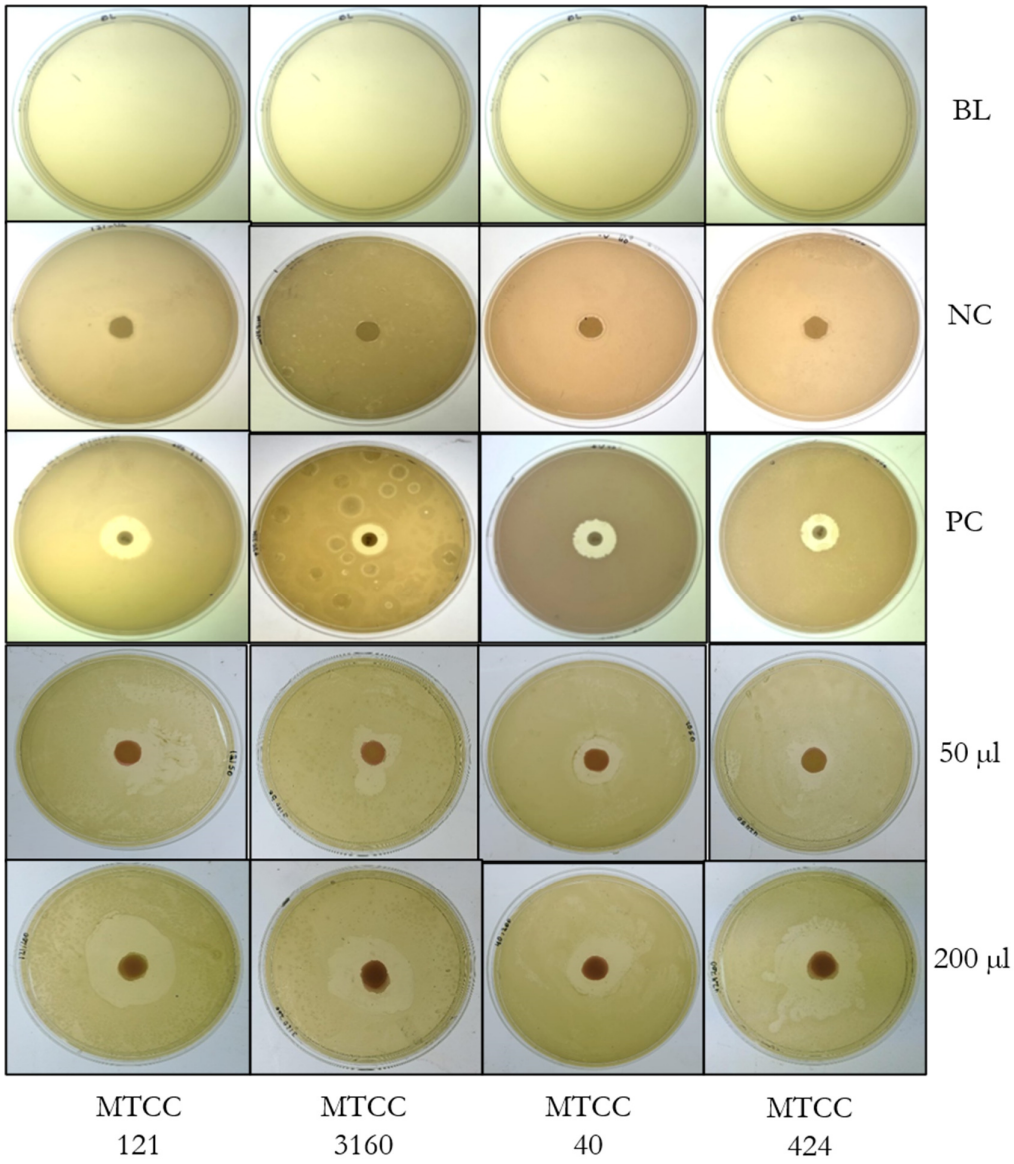


Figure 6. Anti-microbial activity of REO against MTCC40, MTCC121, MTCC424, MTCC3160
BL: Blank (Only Media), PC: Positive Control (Streptomycin 10mg/disc), NC: Negative Control (Empty Disc).

Membrane integrity assay (cytoplasmic content release CCR)

The antibacterial activity of RNE was further verified using the membrane integrity test (CCR). Membrane integrity of RNE was studied in MTCC 40, MTCC 424, MTCC 121, and MTCC 3160 by exposing to 200 µl REO from 0-12 hours (Figure 7). REO depicted antibacterial activity in test pathogen dependent manner. Further, a linear increase in %CCR was observed in the REO-treated all bacterial cells. RNE treated MTCC 424 cells released 100% cytoplasmic content after 10 h of incubation. Whereas MTCC 121 and MTCC 40 exhibited 93% and 90% CCR after 10 h of incubation. When compared to positive control data, it was observed that RNE exhibited higher CCR to reference control Triton-X-100 which exhibited CCR in the range of 48-66%. Major processes including osmoregulation, transport, lipid synthesis, and peptidoglycan cross-linking are carried out by the bacterial cell membrane. According to Daima *et al*. (2014), the integrity of the bacterial membrane is crucial for survival and can directly or indirectly result in cell death.

Natural oils and their components act against a variety of targets, particularly the cytoplasm and cell membrane, and occasionally completely alter the morphology of the cells, according to studies in the literature on the disruption of membrane cells by essential oils from various medicinal plants (Jerobin*et al.*, 2015; Nazzaro *et al.*, 2013).

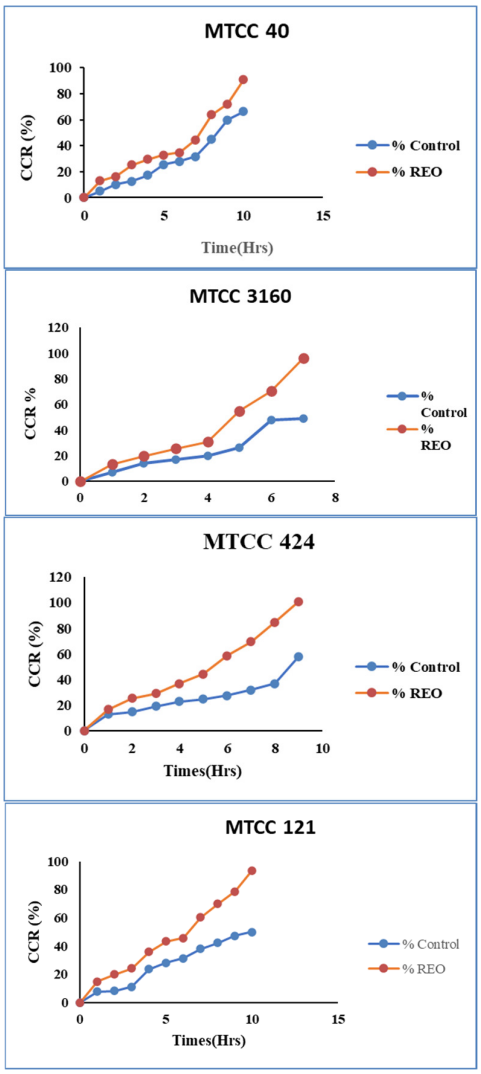


Figure 7. Percentage cytoplasmic content release (CCR) from different bacterial strains treated with pure REO

Anti-fungal activity of REO

The antifungal activity of REO was quantitatively assessed in terms of mycelial growth inhibition (MGI) against *Aspergillum flavus* (MTCC 277), *Aspergillus fumigatus* (MTCC 343) and *Mucor* spp. (MTCC 3373). Two different REO volumes i.e. 50 µl and 200 µl were tested against fungal pathogens (Table 5, Supplementary Figure 1). At 50 µl, incubation time dependent changes in MGI were observed. For instance: against all pathogens, MGI values ranged from 45-71% after 3 days of incubation, however, with passage of time up to 9 days of incubation MGI values reduced to 0%. Notably, by using 200 µl REO, 100% inhibition of mycelial growth was observed in all fungal pathogens from 3-9 days of incubation. Interestingly, at 200 µl after 5-7 days of incubation, MGI was significantly higher than synthetic drug ketoconazole (positive control),commonly

used as a treatment for dermatological fungal infections. After 9 days of incubation, synthetic drug almost failed to inhibit mycelial fungal growth of all test pathogens. Anti-fungal potential of REO may be due to the presence of major component and minor bioactive compounds *in toto*. Earlier study by Mertas *et al.* (2015) also reported high level of terpinen-4-ol in essential oils behind its antifungal activity. Substantial antifungal activity of REO has also been pronounced for other pathogenic fungus like *Sporothrix brasiliensis, Fusarium verticillioides* (Waller *et al*., 2021; da Silva *et al*., 2015). It is possible that REO easily enters fungal cell wall thus cause membrane leakage of electrolytes or lipid peroxidation of membrane which eventually hinders fungal hyphal-growth and germination of conidia of fungal strains (Bakkali *et al*., 2008). It was also noted that some antifungal agents inhibit fungal growth by intruding ergosterol biosynthesis through binding to ergosterol in membranes. This damages the integrity and function of membrane-bound proteins and disturbs osmosis, fungal growth and proliferation (Bendaha *et al*., 2011).

Table 4. Antibacterial activity of REO

Microorganism	MTCC	Strain type	Zone of inhibition (cm)		
			REO [50 µl]	REO [200 µl]	PC Streptomycin (10 mg/ml)
Pseudomonas aeruginosa	424	Gram negative	0.2±0.01a	1.7±0.02a*	0.3±0.02a
Escherichia coli	40	Gram negative	1.3±0.04b	2.0±0.03b*	0.3±0.01a
Bacillus subtilis	121	Gram positive	1.0±0.05b	2.5±0.02c*	0.8±0.03b
Staphylococcus aureus	3160	Gram positive	0.3±0.02a	2.1±0.02b*	1.1±0.04c

Table 5. Antifungal activity of REO against different fungal strains

Fungal culture	Sample	REO volume/ (µL/25 ML)	Mycelial growth inhibition (%)			
			Day 3	Day 5	Day 7	Day 9
MTCC 277 *Aspergillus flavus*	NC	0	9	0	0	0
	REO	50	45	0	0	0
	REO	200	100	100	100	100
	PC	Ketoconazole (25 µg/ml)	98	61	50	50
MTCC 343 *Aspergillus fumigatum*	NC	0	4	0	0	0
	REO	50	61	0	0	0
	REO	200	100	100	100	100
	PC	Ketoconazole (25 µg/ml)	94	23	0	0
MTCC 3373 *Mucor* spp.	NC	0	4	0	0	0
	REO	50	71	0	0	0
	REO	200	100	100	100	100
	PC	Ketoconazole (25 µg/ml)	98	72	70	70

NC: Negative control, PC: positive control, REO: Rosemary essential oil

Conclusions

GC-FID analysis confirmed that the *Rosmarinus officinalis* essential oil possesses a considerable amount of bioactive compounds compounds like Terpinen-4-ol, 1,8 cineole, β-Pinene, α- and other minor components and can be used in pharmaceutical due to its capability of antioxidant and anti-inflammatory. High concentrations of phytochemicals like tannin (1.309 mg AA/ml), flavonoids (0.056 mg RUE/ml), and phenolics (0.653 mg GAE/ml) were detected in GEO. The IC_{50} value of this oil for various antioxidant assays was: DPPH (101), ABTS (172), iron chelating (32), hydroxy radical scavenging activity (16). *Rosmarinus officinalis* essential oil showed anti-inflammatory and inhibits the alpha-amylase in a competitive manner. Maximum antibacterial activity was observed of *Bacillus subtilis* with ZOI 2.5 cm. High potential of REO in performing, anti-inflammatory, anti-diabetic activities was revealed in dose dependent manner. REO performed well to mitigate growth of G^+ and G^- bacteria. Substantial antifungal activity against fatal fungal disease like Aspergillosis and Mucormycosis was also revealed. This study suggests that REO has high potential to be used as an important raw material in pharmaceutical, food, and cosmetic industries.

Authors' Contributions

ADS: design and MS write up, IJ: MS write up, JK: wet lab, AC: wet lab
All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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