Chapter 8

Antimicrobial and Antioxidant Properties of Essential Oil Isolated from *Coleus zeylanicus* under Normal and Salinity Stress Conditions

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Abstract

Essential oils can be used as antibacterial additives and are generally recognized as safe. *Coleus zeylanicus* is one of the medicinal aromatic plant serves as a source of essential oils. Antimicrobial and antioxidant activities of essential oils obtained from the control and salinity stressed *Coleus zeylanicus* plant was investigated in the present study. Essential oils from the control and salinity stressed *Coleus zeylanicus* plant was extracted using Clevenger apparatus. The composition of essential oils was identified using gas chromatography mass spectrometry, which showed a few compounds expressed differentially. The antibacterial activity of the isolated essential oils was studied by using the agar well diffusion method, showing potent inhibitory activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The antioxidant and antimicrobial constituents of the essential oils were spotted using the bioautography method, revealing that the antioxidant and antimicrobial properties in the essential oils of *Coleus zeylanicus* were increased upon exposure to salinity stress.

Keywords: agar well diffusion method, salinity stress, essential oils, bioautography method, thin layer chromatography

1. Introduction

In the recent years, the increasing resistance and the wide spread of multi-drug resistant microbes are leading to a serious health problem to the human population. The emergence of resistant microbes is due to the indiscriminate use of antibiotics [1]. Hence there is a need

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to identify new drugs with effective antimicrobial and antioxidant properties to overcome this problem and to replace the usage of synthetic drugs responsible for the cause of side effects in patients. The resistance to antibiotics can be reduced by the use of resistance inhibitors isolated from plants. Salt stress is a vital abiotic stress factor that affects the growth and productivity of plants. In general, salinity refers to the presence of different salts like sodium chloride, calcium sulfate, magnesium, and bicarbonates in water and soil [2]. The uptake of water and absorption of essential nutrients by plants are restricted due to the presence of soluble salts exerting high osmotic pressure which ultimately affects the growth of plants [3]. Plants have adopted a mechanism to tolerate salt stress by the accumulation of solutes such as glycine, betaine, proline, sugar alcohols, polyols, and soluble sugars and by eliminating the toxic Na^+ ions in the cytoplasm [4]. Plant bioactive compounds are low molecular weight secondary metabolites distributed largely in plants that play a major role in the adaptation of plants to different environmental changes and in overcoming stress constraints. Medicinal plants exhibit pharmacological properties as they are known to possess various bioactive compounds called secondary metabolites like tannins, terpenes, alkaloids, steroids, flavonoids, glycosides, saponins, etc. These compounds play a major role in protecting the plants from various stress factors. Secondary metabolites that act as active components exhibit a wide range of antimicrobial activity [5]. The target sites shown by plant extracts are active against drug resistant microbes than those used by the antibiotics. It was discovered that the non-antibiotic substances such as essential oils have shown good fighting potential against drug resistant microbes. Essential oils change the rate of an enzyme reaction by interfering with the metabolism of microbes; thereby influencing the uptake of nutrients from the medium and affect the synthesis of enzymes or by changing the membrane structures that leads to the death of microbes. Thus, with the discovery of many natural products from plant species due to advancements in science and technology made a remarkable progress in the field of medicine. Now-a-days, many researchers are interested in isolating the biologically active compounds from plant species for developing the novel drugs in order to combat the microbes responsible for dreadful diseases [6].

Essential oils are a heterogeneous group of complex mixture of organic compounds synthesized within plants as secondary metabolites with characteristic flavor and odor. The quantity and quality of oil vary depending on the ecological and growth conditions of plant chosen for extraction. The various other factors also influence the yield of essential oils. The different parts of the plant such as leaves, root, stem, seeds, bark, woods, twigs, buds, fruits, and flowers can be used for the extraction of aromatic oily liquids called essential oils [7]. Essential oils are a mixture of compounds principally terpenoids like (C_{10}) monoterpenes, (C_{15}) sesquiterpenes, and (C_{20}) diterpenes, also contains lactones or acyclic esters, low molecular weight aliphatic hydrocarbons, aldehydes, alcohols, acids and rarely may contain coumarins, nitrogen-, and sulfur-containing compounds and homologs of phenylpropanoids [8]. Essential oils are produced commercially by the method of steam distillation; whereas the other methods of extraction, fermentation, and expression can also be performed to obtain oils [9]. Essential oils possess antibacterial, antifungal, antiviral, anticancer, antioxidant, and insecticidal properties [10]. Essential oils from medicinal plants possess antioxidant activity, which plays an important

role in neutralizing free radicals benefiting human health. Essential oils also showed a potent inhibitory effect against Gram-positive bacteria, Gram-negative bacteria, filamentous fungi, and yeast. The highest inhibitory activity of *C. zeylanicus* oil against a wide spectrum of bacteria and fungi was reported [11].

Coleus zeylanicus is a perennial aromatic herb belonging to the family of *Lamiaceae*. It has astringent and stomachic properties used in the treatment of fever, common cold, asthma, dysentery, diarrhea, vomiting, burning sensation, small pox, eye diseases, worm diseases, chronic ulcers, dental diseases, and thirst [12]. The juice obtained from the stem and leaves of *C. zeylanicus* are used to treat diarrhea when taken along with honey [13]. It acts as diuretic, diaphoretic, and cholagogue which are useful for chronic and acute congestion of the liver. *C. zeylanicus* is also used to develop potential biodegradable micro-biocides [14]. The medicinal properties of this plant were moderately understood, but not much work was done on the properties of the essential oils isolated from the leaves exposed to salinity stress. In the present study, essential oils were isolated from the control and salinity stressed leaves of *Coleus zeylanicus* and their compositions were determined using gas chromatography mass spectrometry. Antibacterial and antioxidant properties of these essential oils were studied.

2. Material and methods

2.1. Plant material and salt stress treatment

Coleus zeylanicus plants (Herbarium specimen no. 21904) were propagated in the GITAM University botanical garden in 12 inch pots under 720 min natural photoperiod [irradiance (400–700 nm) of 1600–1800 μmol m^{-2} s^{-1}] with day/night temperatures of 30/23°C with an approximate air humidity of 60%. The pots were arranged in rows 1 m apart and the plants were irrigated daily and fertilized weekly with Hoagland solution. Three month old plants with uniform growth were selected for this study. Plants were then separated into two groups. Control plants were watered daily and salt stressed plants were treated with 250 ml of 300 mM NaCl solution twice a day for a period of 1 week. The plant materials were dried separately in shade and powdered, which were used for the extraction of essential oils.

2.2. Extraction of essential oil

The essential oils were extracted using Clevenger apparatus. Distillation is carried out for a period of 4 h by immersing the dried leaf powder directly into a round bottom flask filled with water. The contents were boiled and the vapors were condensed, allowing the essential oils to separate based on their difference in immiscibility and density. After extraction, the organic phase was separated and dried over Na_2SO_4. The percentage of the oils was calculated using the following formula:

Oil (% v/w) = observed volume of oil (ml)/weight of sample (g) × 100.

2.3. Identification of compounds using GC-MS

The identification of the major chemical composition of the isolated essential oil was performed by GC-MS (Sathyabama University, Chennai). The extracted solution containing essential oil was separated using diethyl ether and the injected sample volume was 1.0 μl for control and 0.5 μl for the salt stress sample as it was more concentrated. A Shimadzu GC-MS QP2010, a polyethylene glycol (Carbowax), and model Rtx-Wax (RESTEC) (30 m to 0.25 mm i.d., film thickness 0.25 lm) capillary column were used for the analysis. The temperature was first held at 40°C and then raised to 250°C (10 min, 2°C/min). The carrier gas was helium at a flow rate of 3 ml min^{-1}. The components of the oil were identified based on the comparison of their retention indices and mass spectra with the fragmentation patterns for computer matching with the NIST (National Institute of Standards and Technology) library.

2.4. Antimicrobial studies of the isolated essential oils

2.4.1. Microorganisms used

Three bacterial strains, *Escherichia coli* (MTCC 1652), *Staphylococcus aureus* (MTCC 3160), and *Pseudomonas aeruginosa* (MTCC 1688), were used in the present study obtained from Microbial Type Culture Collection Centre, Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.4.2. Preparation of inoculum

The colonies of test organisms were inoculated into 0.85% normal saline and the turbidity adjusted to 0.5 McFarland using the standard, which is equal to 1.5×10^8 CFU/ml.

2.4.3. Antibacterial activity by the agar well diffusion method

The antibacterial activity of the essential oils isolated from *Coleus zeylanicus* control and salinity stressed plants was analyzed by an agar well diffusion method. The essential oil was tested against the selected bacterial strains *E. coli*, *S. aureus*, and *P. aeruginosa*. Sterile Muller Hinton agar medium was poured into each Petri dish and allowed to solidify. After solidification, culture was spread over the plate by a spread plate technique using sterile cotton swab. Wells of 5 mm size were made in the agar plates with the help of sterile cork borer; the wells were then loaded with 200 μl of sample (essential oil dissolved in solvent), solvent alone as negative control and antibiotic as positive control. All the plates were incubated at 37°C for 24–48 h. After incubation, the plates were observed for the zone of inhibition around the well [15]. The zone of inhibition was calculated by measuring the diameters of the inhibition zone around the well.

2.5. Identification and separation of compounds using TLC

The essential oils obtained by the method of steam distillation are subjected to thin layer chromatography to identify and separate the bioactive compounds present in both the samples of

control and salt stress. Both the control and salt stress samples were applied to the TLC plate separately. The solvent system used in the TLC analysis was toluene:ethyl acetate in the ratio of 93:7. TLC was carried out using TLC silica gel 60 F_{254} aluminum sheets (Merck). After complete elution, the spots were identified and R_f values were calculated for each spot.

2.6. Screening of antioxidant activity

Several TLC techniques have been developed successfully for the analysis of antioxidants both quantitatively and qualitatively. The use of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical reagent for the analysis of antioxidant activity by the TLC method is one among them. TLC bioautography assay for screening of antioxidants possess several advantages that include high throughput, simplicity, and flexibility. In the present study, the antioxidant activity of essential oils is evaluated using the method of TLC bioassay. The components of essential oils were separated on the TLC plate and sprayed with DPPH solution. Antioxidant reduces the radical and produces creamy spots against a purple background.

2.7. Screening of antimicrobial activity

To screen the antibacterial activity of essential oils from control and salt stress, the method of direct bioautography is performed. Initially, the components were separated on the TLC plate and allowed to air dry. The test organisms were allowed to grow on the TLC plate by dipping the plate into the respective medium containing the organism, followed by incubation at 37°C for 24 h. After incubation, the plates were sprayed with 2 mg/ml solution of p-iodonitrotetrazolium violet dye. A clear zone indicated the inhibition of growth of the organism.

2.8. Statistical analysis

Results mentioned are reported as the mean ± standard error (SE) values of five independent experiments, conducted on five different plants in each experiment. SE values were calculated directly from the data according to standard methods. Data analyses were carried out using the SPSS package. Mean values were compared by Duncan's multiple range test and P-values which are less or equal to 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Essential oil analysis

The essential oils obtained by the hydrodistillation of *Coleus zeylanicus* leaf powder under control as well as salinity stress conditions was 1% (v/w) and 0.93% (v/w), respectively, based on the gram dry weight of the leaf powder. Analysis of the essential oil was carried out using GC-MS, which identified a total of 14 compounds in the essential oil isolated from the control *Coleus zeylanicus* plants and 7 compounds from the oil isolated from the *Coleus zeylanicus* leaves exposed to 300 mM salinity stress (**Figures 1** and **2**). Majority of the compounds present

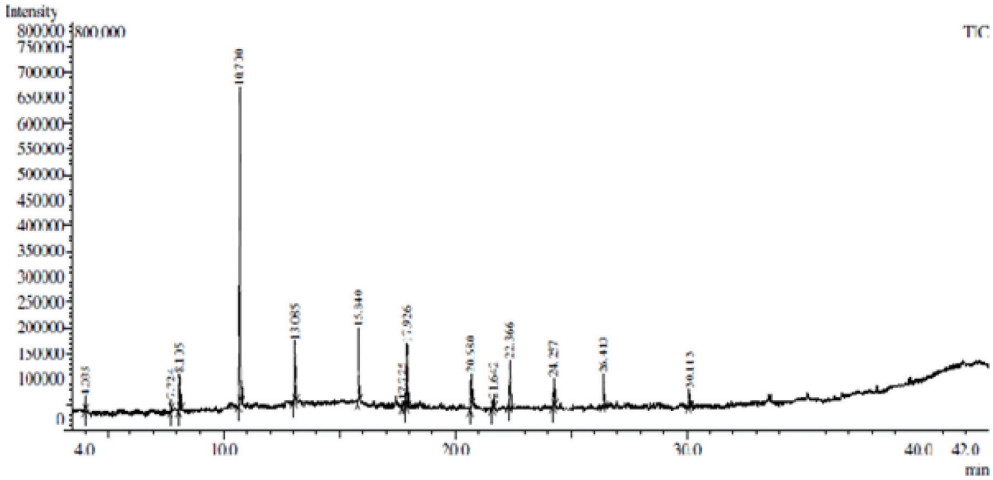

Figure 1. GC-MS result of essential oil obtained from *coleus zeylanicus* control.

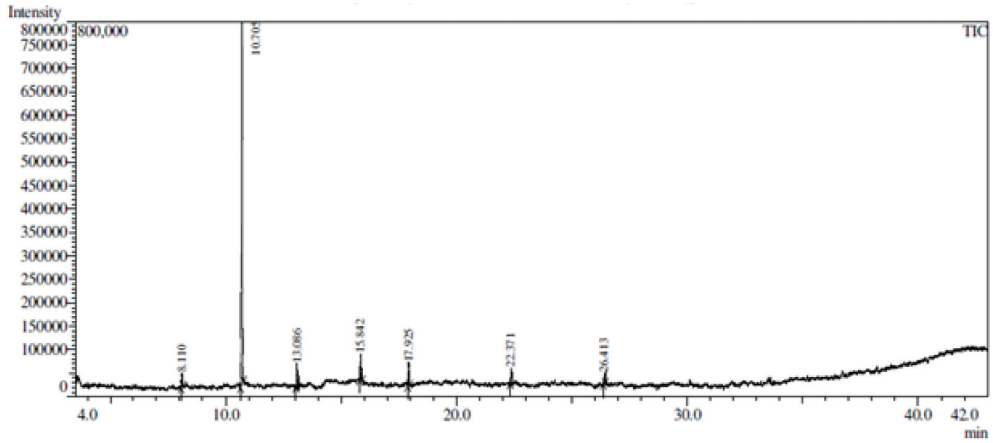

Figure 2. GC-MS result of essential oil obtained from *coleus zeylanicus* salinity stress plant.

in the essential oil of *Coleus zeylanicus* under control conditions was not identified in the essential oil extracted from the leaves exposed to the salinity stress, which made the essential oil composition of the salinity stressed *Coleus zeylanicus* different from that of *Coleus zeylanicus* plant growing under control conditions (**Tables 1** and **2**). Only compounds that were identified to be common in both the essential oils were Z-5-nonadecene and 2,4-di-tert-butylphenol. Thymol and carvone are found to be present in higher percentages of control and salinity stressed essential oils of *Coleus zeylanicus*. These two terpenoids were the major components of the essential oil extracted from *Lippia* species showing antiprotozoan properties [16]. The percentage of Z-5-nonadecene and 1-nonadecene components was decreased in the salinity stress treated essential oil of *Coleus*

zeylanicus. The difference in the composition of essential oils might be due to salinity stress [17]. Long chain fatty acids 1-heptadecene and 1-nonadecene isolated from the root ethanol extracts of *S. longepedunculata* possess antifungal and antibiotic properties [18, 19]. In the present study, the essential oils extracted from the *Coleus zeylanicus* leaves during control and salinity stress had a different chemical composition, which were tested for their antimicrobial and antioxidant properties.

3.2. Antimicrobial activity

The antibacterial activities of essential oils extracted from the control and salinity stressed *Coleus zeylanicus* leaves were tested against *E. coli*, *S. aureus* and *P. aeruginosa*, which have shown moderately high inhibition (**Table 3**). The antimicrobial susceptibility testing was done using the disc diffusion method. Essential oils showed effective inhibitory activity against *E. coli* than that of *S. aureus* and *P. aeruginosa* with zone of inhibition increasing from 12 to 16 mm, respectively. The essential oil of *C. furcata* showed inhibition zones of 14 and 13 mm against the Gram-negative strains of *E. coli* and *P. aeruginosa*; 14 mm against the Gram-positive strain *S. aureus* [20]. The oxygenated monoterpenes exhibit strong antimicrobial activity than hydrocarbon derivatives as they are less water soluble limiting the diffusion through medium. Essential oils containing hydroxyl group exhibits strong antimicrobial activity which might be due to the capability of binding easily to the enzyme at the active site and altering their metabolism. The combination of electrolyzed NaCl and essential oil with 0.5% carvacrol and 0.5% thymol reported to be effective in controlling microbial population and chemical deterioration

Peak No.	R. time	Area	Area (%)	Name of the compound
1	4.035	44,267	1.29	1-Decene
2	7.724	45,372	1.32	endo-Borneol
3	8.105	147,076	4.28	1-Dodecene
4	10.700	1,366,605	39.74	Thymol
5	13.085	261,154	7.59	9-Octadecene
6	15.840	353,293	10.27	2,4-Di-tert-butylphenol
7	17.725	69,757	2.03	(−)-Caryophyllene oxide
8	17.926	296,236	8.61	1-Heptadecene
9	20.680	180,412	5.25	Dibenzo[a,h]cyclotetradecene
10	21.642	48,642	1.41	6-Methyl-5-(1-methylethylidene)
11	22.366	235,008	6.83	Z-5-Nonadecene
12	24.257	143,316	4.17	(−)-Isolongifolol, methyl ether
13	26.413	159,508	4.64	Behenic alcohol
14	30.113	87,997	2.56	1-Heptacosanol

Table 1. Composition of the essential oil extracted from the *Coleus zeylanicus* control plant.

Peak No.	R. Time	Area	Area (%)	Name of the compound
1	8.110	55,347	2.14	3-Tetradecene
2	10.705	2,000,267	77.23	Carvone
3	13.086	129,091	4.98	1-Pentadecene
4	15.842	147,692	5.70	2,4-Di-tert-butylphenol
5	17.925	119,179	4.60	E-14-Hexadecenal
6	22.371	82,567	3.19	Z-5-Nonadecene
7	26.413	55,879	2.16	1-Nonadecene

Table 2. Composition of the essential oil extracted from the salinity stressed *Coleus zeylanicus* plant.

Strain name	*C. zeylanicus* control (mm)	*C. zeylanicus* salt stress (mm)	Positive control (ampicillin) (mm)	Negative control (solvent) (mm)
S. aureus	17 ± 2.87	17 ± 2.82	32 ± 2.65	No zone
E. coli	12 ± 2.10	16 ± 3.12	No zone	No zone
P. aeruginosa	17 ± 2.75	17 ± 1.80	No zone	No zone

Table 3. Antimicrobial activity of both the essential oils by agar well diffusion method.

[21]. Essential oils act indirectly on membranes by secreting toxins which play an important role in controlling the microbial population of *S. aureus* and *B. cereus*. These essential oils can also be used in combination with other antibacterial agents to enhance their activity. Addition of lysozyme enhanced the synergistic activity between carvone and nisin [22]. The antimicrobial activity of essential oils depends on the chemical composition and the amount of single compound present. These compounds occur in the active form in plants or can be activated by specific enzymes when subjected to biotic or abiotic stress. Mechanism of antimicrobial activity of the essential oils includes the damage of the cytoplasmic membrane, degradation of cell wall, decreased ATP synthesis, membrane protein damage, reducing the proton motive force, and increasing the membrane permeability by reducing the membrane potential [23]. In the present study, the antibacterial activity of the essential oil of *C. zeylanicus* under both the conditions of control and salinity stress was effective against the tested strains. It was also observed that the activity was enhanced when the plant is subjected to salinity stress.

Majority of the essential oils has shown effective inhibitory activity against Gram-positive strains [24], might be due to the presence of hydrophilic outer membrane, which prevents the entry of hydrophobic compounds into the target cell membrane, thereby acquiring resistance to the antimicrobial drugs [25]. Another possible reason could be the inhibition of microbial respiration and increased membrane permeability by essential oils resulting in the death of microbes after massive ion leakage [26, 27]. Therapy with traditional herbs is practiced with the plant species containing medicinal properties. Secondary metabolites such as terpenoids, tannins, alkaloids, phenols, and flavonoids rich in plants are found to be responsible for

antimicrobial properties *in vitro*. Plant phytochemicals serve in defense mechanisms against predation by herbivores, insects and microorganisms. Quinones and tannins were responsible for plant pigment, whereas terpenoids responsible for plant odor and flavor. Highly oxygenated phenols were found to be highly toxic to microorganisms. The search for new compounds with antimicrobial property derived from natural plant sources has gained much attention to replace synthetic drugs. The growth of microbes can be controlled with the use of phytochemicals derived from plant source which are more effective and less toxic [28, 29].

3.3. Screening of antioxidant and antimicrobial activity by the TLC bioautography method

Bioautography technique was employed to detect the antimicrobial and antioxidant activity [30]. In our study, the two essential oils spotted on the TLC plate were separated into distinct bands with different R_f values. Essential oil from control and salt stressed *C. zeylanicus* have characterized by the presence of three antimicrobial compounds with the R_f values of 0.34, 0.54 and 0.72. The developed TLC plates were used separately for determining the presence of antimicrobial and antioxidant compounds present in the essential oils of *Coleus zeylanicus.* The antioxidant activity of the compounds present in the essential oil was also determined by developing the TLC plate with 20 μg/ml of DPPH solution after the separation of compounds (**Figure 3**). Similar results were reported with the essential oil of *Eucalyptus lanceolatus* [31]. By using the agar overlay bioautography method, several antimicrobial compounds were identified and isolated from the husk, cotyledons and tubers of *Tylosema esculentum* [32].

Figure 3. The antioxidant activity of the compounds present in the essential oil determined by developing the TLC plate with 20 $\mu g/ml^{-1}$ of DPPH. Lane 1: essential oil isolated from control leaves of *C. zeylanicus*. Lane 2: essential oil isolated from salinity stressed leaves of *C. zeylanicus*.

In this method of TLC bioautography, a developed TLC plate is dipped into the respective broth containing microbes of pure culture and incubated under humid conditions. The microbes grow directly on the TLC plates except in the regions where the bioactive compounds exhibit the antimicrobial property. The zones of inhibition with creamy spots against purple background are visualized after spraying the plates with INT (p-iodonitrotetrazolium violet) dye. The tetrazolium salts are converted into a purple colored compound called formazan by the dehydrogenase activity of living microbes. Once the activity is located on the TLC plate, the samples can be analyzed by GC-MS to identify the presence of known or unknown compounds responsible for the activity [33]. This method is considered to be convenient for obtaining the reliable information on the activity of single compounds as the plant extracts possess numerous bioactive compounds. The analytical determination of compounds present in plant extracts and the characterization of their biological properties are made possible with the optimized antimicrobial assays. Separation of compounds in plant extracts is necessary to avoid study on fractions with no biological activity. Detection of antimicrobial compounds by this method is rapid, uncomplicated and effective in saving money and time [34]. Apart from the search of bioactive compounds, this method is also used to find out best solvent for the extraction of compounds and for the selection of mobile phase to separate compounds. It was reported that the thymol and carvacrol were responsible for the antimicrobial property present in the essential oils of *T. vulgaris* L. using the dot blot test [35].

The TLC bioautography method also used to detect the compounds exhibiting antioxidant activity. The developed TLC plate sprayed with DPPH (2,2-diphenyl-1-picrylhydrazyl radical) solution produces clear creamy yellow spots against a purple background. The DPPH decreases upon the reduction reaction with a radical scavenger leading to the color change which can be observed in a TLC bioassay. The reaction has been depicted in **Figure 3**. The assay depends on the measurement of antioxidants scavenging activity, where the DPPH is characterized as a stable free radical. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants present in the plant extracts to the corresponding hydrazine. Rosmarinic acid, luteolin, chrysoeriol and apigenin were the four different antioxidant compounds isolated from the fruit of *Perilla frutescens var. acuta* by a TLC bioautography method using DPPH as a detection reagent [36].

4. Conclusion

The results of this study showed that the essential oils of *C. zeylanicus* showed good antibacterial activity against the tested pathogenic strains. The essential oils containing compounds with antimicrobial and antioxidant activity were identified by the method of bioautography technique. Chemical profiling by GC-MS showed thymol and carvone as major components in control and salt stressed essential oil. The present study mainly focused on to observe the change in the level of bioactive compounds in the essential oils of *coleus zeylanicus* when the plant is subjected to salinity stress for determining its commercial value. Our study concludes that the antimicrobial and antioxidant activity remained to be effective even under stress conditions.

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