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Research article

Assessments of Chemical Constituent and Antimicrobial Activity in *In Vitro* Cultures of *Coleus forskohlii*

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Abstract

The aim of the current research was to explore the chemical composition and antimicrobial efficacy of several extracts derived from *in vitro* calli and plantlet cultures of *C. forskohlii*. According to the findings, C1 treatment (1.0 mg/L 2,4-D + 0.5 mg/L Kin) was the most suitable for inducing friable calli in both explants (leaf and root). Regarding the plantlet culture, it was discovered that using MS medium supplemented with 0.5 mg/L Kin was preferable for the multiplication of shoots. It was also noted that 0.5 and 1.0 mg/L cytokinin treatments recorded the maximum rooting percentage (100%). HPLC identified 14 phenolic and flavonoid compounds in both the aqueous and methanol fractions. The major component was gallic acid, which had the highest concentrations (23.43 and 23.62 µg/g DW) in the calli of root and leaf, respectively, in 80% methanol extract and 37.0 µg/g DW in aqueous extract of plantlet culture. According to GC-MS analysis of inorganic extracts (chloroform and ethyl acetate), fatty acid compounds appeared to be the main components in the chloroform fractions of different extracts. The most prevalent was 9,12-octadecadienoic acid, methyl ester, at rates of 16.33%, 11.69%, and 25.11% in callus culture from root, and leaf, and in plantlet culture, respectively. For ethyl acetate extracts, 1-butanol-3-methyl acetate (isoamyl acetate) was the most abundant compound in the extracts, recorded at 48.29%, 44.90%, and 46.06% in callus culture from the roots and leaves, and from plantlet culture, respectively. The antimicrobial activity of the various extracts revealed that the methanol extract was the most effective, inhibiting the growth of all pathogenic microorganisms when tested at 300 µg/mL, and demonstrating broad visibility efficacy against bacteria and molds, with MIC ranging from 125 to 260 ppm.

Keywords: *Coleus forskohlii*; callus culture; plantlet cultures; chemical constituents; antimicrobial agent

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1. Introduction

Coleus forskohlii (Lamiaceae), first discovered in India, is one of the essential species of the genus *Coleus* that has a strong odor like camphor. It is grown in a high-density area of more than 2500 hectares and is recognized as the most economically wealthy plant (Valdes et al., 1987; Soni & Singhai, 2012). It has been used to produce some ancient drugs from roots and has been utilized in the treatment of many disorders, including heart diseases, abdominal colic, respiratory disorders, insomnia, convulsions, asthma, bronchitis, intestinal disorders, burning sensation, constipation, epilepsy, and angina (Ammon & Müller, 1985).

Important secondary metabolites like forskolin, rosmarinic acid, flavonoids, tannins, reducing sugars, alkaloids, and essential oils like thymol and carvacrol are present in many *Coleus* species, including *Coleus forskohlii* (Bhat et al., 1977). Diterpenoids and essential oils have been reported as fundamental compounds in the plant (Pino et al., 1989). Flavonoids are a group of polyphenolic compounds that exert direct health-promoting effects such as antimicrobial, anti-inflammatory, antioxidant, antiallergic, and anti-cancer properties (Aiyelaagbe & Osamudiamen, 2009; Alasbahi & Melzig, 2010).

Phenolic compounds like gallic acid have many biological activities, including antibacterial, anti-neoplastic, anti-melanogenic, and anti-oxidant activities (Kim, 2007). Essential oils or some of their constituent parts are used in a variety of products, including perfumes and cosmetics, sanitary products, dental work, agriculture, food additives, and natural remedies (Bakkal et al., 2008). This effect is mostly brought on by the terpenes and terpenoids found in essential oils. Numerous researches have discussed the antibacterial properties of specific components and essential oils (Burt, 2004; Ait-Ouazzou et al., 2011).

Experiments on the antimicrobial activity of plant extracts under various conditions have shown that the extracts can cause lethal effects at lower doses, providing a suitable solution to the problem of bacterial resistance to antibiotics (Rodrigues et al., 2009; Alaribe et al., 2011). *Coleus forskohlii* root extracts have previously demonstrated antimicrobial activity against a wide range of microbial species, including bacteria such as *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Sericea*, *Kelebsiella pneumonia* and *Bacillus pumilus*, and fungi: *Aspergillus flavus*, *Aspergillus parasiticus*, *Trichoderma rubrum* and *Microsporum gypseum* (Saklani et al., 2011). Additionally, phenolic compounds from *Coleus forskohlii* showed antibacterial action against Gram-positive and Gram-negative bacteria and yeast, *Candida albicans* (Shaker et al., 2022).

Recently, it has been possible to produce therapeutic components *in vitro* under carefully regulated settings using plant tissue culture techniques. A plant growth regulator concentration is one of the most restricting elements regulating cell growth, differentiation, and metabolite synthesis (Liang et al., 1991).

Through the callus and plantlet culture of *Coleus forskohlii*, we aimed to create potent plant extracts that could be used as antibacterial agents in our work. Furthermore, the chemical make-up of these extracts was investigated to determine the most important substances responsible for their effectiveness.

2. Materials and Methods

2.1 Plant material

Coleus forskohlii seeds were cleaned with tap water that was currently available, surface sterilized for 30 s in 70% (v/v) ethanol, and then submerged for 15 min in 50% Clorox solution (5.25% sodium hypochlorite) with a drop of Tween-20. The seeds were cultivated on basal MS medium (Murashige & Skoog, 1962), supplemented with 0.7% (w/v) agar and 3% (w/v) sucrose, following a thorough four-time washing in sterile water. A regulated light regime (16/8 h photoperiod and 2,000 lux) was used to incubate the cultures at 25±1°C. Subsequently, *in vitro* seedlings were used as plant material.

2.2 *In vitro* callus cultures

Leaf and root explants were excised from one-month *in vitro* seedlings and cultured on a solidified MS basal nutrient medium supplemented with different growth regulators as follows:

C1 (1.0 mg/L 2,4-D + 0.5 mg/L Kin)
C2 (2.0 mg/L 2,4-D +2.5 mg/L Kin)
C3 (1.0 mg/L NAA + 0.5 mg/L BA)
C4 (2.0 mg/L NAA + 2.5 mg/L BA)

After one-month of culturing, callus initiation frequency was recorded and calculated based on the following equation:

Callus initiation frequency % = (Number of initiated calli)/ (Number of inoculated explants) × 100.

C1 medium, which was found to be the best one, then was used for calli sub-culturing for three passages, one month each.

2.3 *In vitro* plantlet formation and multiplication

Explants of shoot tips were taken from growing seedlings, and cultured on a solidified MS basal nutrient medium supplemented with BA (0.5,1.0,2.0 mg/L) and Kin (0.5,1.0,2.0 mg/L). After one month, the shoot length, number of leaves, and number of shoots/explants were recorded and calculated. The rooted shoots were also observed and calculated on the same media. The formed plantlets were then sub-cultured in the best medium for multiplication.

2.4 Preparation of the extracts

Dried powdered samples of callus and *in vitro* plantlet cultures were extracted using water, methanol (80%), chloroform, and ethyl acetate. Water (aqueous) and methanol (80%) extracts were performed by soaking the powdered plant material in the solvent for 24 h at room temperature. The chloroform, and ethyl acetate extracts were prepared by extraction under reflux for 4 h. All extracts were collected, filtered, and evaporated to dryness. Each residue was dissolved in its original extraction solvent and stored at 4°C. The polar extracts (aqueous and methanol 80%) were subjected to HPLC analysis for phenolic and flavonoid

fractionation and determination, while non polar extracts (chloroform and ethyl acetate) were taken for GC-MS analysis to identify different components.

2.5 Fractionation and determination of phenolic and flavonoid compounds

HPLC was used to separate and identify the phenolic components in both aqueous and methanol (80%) extracts. Agilent Technologies 1100 series liquid chromatograph, which was outfitted with a diode-array detector and an auto sampler. Eclipse XDB-C18 (150 X 4.6 µm; 5µm) analytical column (Phenomenex, Torrance, CA) with C18 guard column was utilized. Acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B) made up the mobile phase. For a total of 70 min, the gradient programmer was set to operate at a flow rate of 0.8 mL/min: 100% B to 85% B min in 30 min, 85% B to 50% B min in 20 min, 50% B to 0% B min in 5 min, and 0% B to 100% min. For the phenolic acid, cinnamic acid derivatives, and flavonoids, peaks were concurrently observed at 280, 320, and 360 nm using a 50 µL injection volume. Prior to injection, each sample was passed through a 0.45 µm Acrodisc syringe filter (Gelman Laboratory, MI). Congruent retention periods and UV spectra were used to identify the peaks, which were then compared to the standards.

2.6 Gas chromatography–mass spectrometric analysis

The non-polar extracts (chloroform and ethyl acetate) were analyzed using GC-MS with the following specifications: a TRACE GC Ultra Gas Chromatograph (THERMO Scientific Corp., USA), coupled with a THERMO mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). Capillary column made of TG-5MS-fused silica (30 m, 0.251 mm, 0.1mm film thickness) was used. For GC-MS detection, an electron ionization system with an ionization energy of 70 eV was used. At a constant flow rate of 1 mL/min, helium gas was used as the carrier gas. The temperature of the injector and MS transfer line was fixed to 280°C. The chemicals were identified by comparing their respective retention times and mass spectra to those of the GC-MS system's NIST, WILLY library data. A percent relative peak area was used to explore the quantification of all discovered components.

2.7 Antimicrobial activity

2.7.1 Strains and microbial media

Gram-positive bacteria (*Bacillus subtilis* NRRL B-94 and *Staphylococcus aureus* NRRL B-313), gram-negative bacteria (*Escherichia coli* NRRL B-3703 and *Pseudomonas aeruginosa* NRRL B-32), fungus (*Aspergillus flutes* NRC), and yeast (*Saccharomyces cerevisiae* and *Candida albicans* NRRL 477) were used. Nutrient agar medium was used in this study for the growth of bacterial strains whereas malt and yeast media were used for fungal and yeast strains, respectively. The media were sterilized by autoclaving at 121°C for 20 min and then used for subculture. Solid media were used for agar-well diffusion assay (Greenwood, 1983).

2.7.2 Procedure

Bacterial strains were cultured on nutrient medium, while fungal and yeast strains were cultured on malt medium and yeast medium, respectively. Broth media contained the same

contents except agar. For bacteria and yeast, broth medium was incubated for 24 h. For fungi, broth medium was incubated for 48 h; with subsequent filtration of the culture through a thin layer of sterile sintered glass G2 to remove mycelia fragments before the solution containing the spores was used for inoculation. One mL of each inoculum was added to 50 mL of agar medium (50°C) and mixed. The agar mixed with inoculum was then poured into 120 mm Petri dishes and allowed to cool to room temperature. Wells (6 mm) were cut in the agar plates using appropriate sterile corkborer. The wells were then filled up to the surface of the agar with 0.1 mL of the experimental samples. The plates were then left on a flat surface and incubated for 24 h at 30°C for bacteria and yeast and 48 h for fungi and the diameter of the inhibition zones was recorded (Greenwood, 1983).

2.7.3 Determination of minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that inhibits the growth of microbes after 24 h of incubation time. The minimum inhibitory concentration (MIC) for *S. aureus*, *B. subtilus*, *E. coli* and *Ps. aeruginosa* was determined by the well diffusion assay method. Samples were tested for MIC determination according to the method reported by Hammer et al. (1999). The culture medium was poured into Petri dishes and maintained at 45°C until the samples were added to the agar. Samples were added using a micropipette at different concentrations with continuous stirring to ensure uniform distribution. Exactly 50 µL of the different bacterial strains were then pipetted using an automated micropipette onto the surface of the solid medium containing the sample. After the bacteria were absorbed into the agar, the plates were incubated for 24 h at 37°C. The MIC was determined as the lowest concentration of samples that caused visible growth inhibition of each bacterium on the agar plate.

2.8 Statistical analysis

Twenty-four explants were used per each treatment. Means and standard errors (SE) were obtained from analysis for each treatment by the use of Microsoft Excel 2010. Data were presented as means±SE. The significant difference between the means was determined using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test at $p<0.05$, using Statistix (version 8.0).

3. Results and Discussion

3.1 *In vitro* callus cultures

During this experiment, different growth regulators were added to MS-medium in order to induce calli from two types of explants (leaf and root). All tested combinations are shown in Table 1. It was discovered that leaf explants produced the highest percentage of callus initiation frequency (84%) when cultured in C1 (1.0 mg/L 2,4-D+0.5 mg/L Kin) treatment. However, in other treatments, this proportion was reduced; it was absent in the C4 (2.0 mg/L NAA + 2.5 mg/L BA) treatment (0%) with leaf explants. Concerning root explants, there was an unexpected and distinct behavior, but they responded positively to all treatments, especially the C1 (1.0 mg/L 2,4-D+0.5 mg/L Kin) and C3 (1.0 mg/L NAA + 0.5 mg/L BA) treatments, which recorded 69% and 72%, respectively. It should be noted that calli color and friability were practically yellowish and friable in the C1 and C2 treatments; however, they changed to green and became more compact in the C3 and C4 media. The

C2 treatment also demonstrated the production of brown calli. It is evident from this finding that the response of leaf explants to produce calli was more potent than the root explants depending on the kind of growth regulators; the C1 treatment was more suitable to create friable calli in both explants (leaf and root). The best medium for mass calli formation (1.0 mg/L 2,4-D + 0.5 mg/L Kin) was used to subculture calli for three passages with one month each (Figure 1).

Table 1. Callus initiation frequency (%) from leaf and root explants of *Coleus forskohlii* cultivated for one month on different growth regulators

Explant	Treatments	Callus Initiation Frequency (%)
Leaf	MS + C1	84±1.10[a]
	MS + C2	27±0.60[d]
	MS + C3	17±1.40[e]
	MS + C4	00±0.00[f]
Root	MS + C1	69±0.88[b]
	MS + C2	30±1.40[d]
	MS + C3	72±1.10[b]
	MS + C4	54±1.70[c]

Note: C1 (1.0 mg/L 2,4-D + 0.5 mg/L Kin), C2 (2.0 mg/L 2,4-D +2.5 mg/L Kin), C3 (1.0 mg/L NAA + 0.5 mg/L BA), C4 (2.0 mg/L NAA + 2.5 mg/L BA). Results are the mean±SE of at least three replicates, one-way ANOVA with least significance differences comparison tests were used at $p<0.05$.

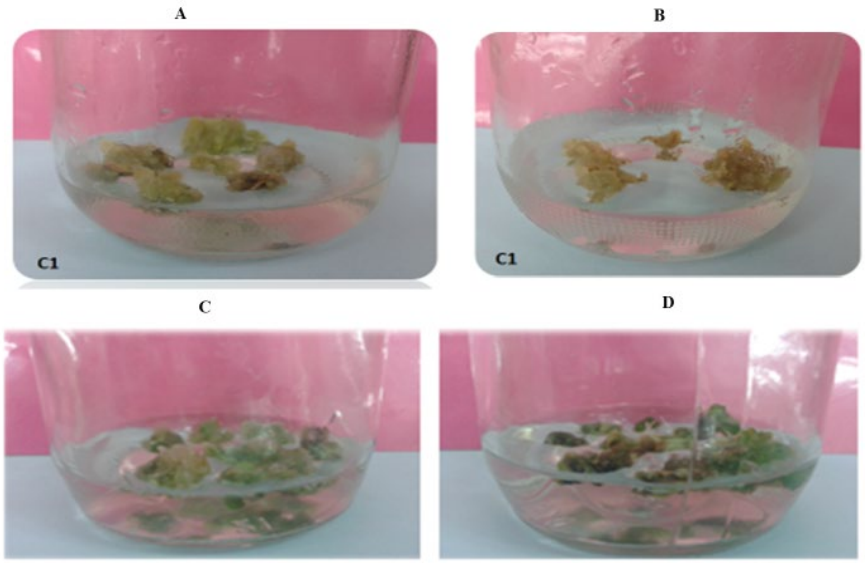

Figure 1. Callus induction from leaf explant (A) and root explant (B) of *Coleus forskohlii* on C1 treatment after one month, calli mass production of leaf calli (C) and root calli (D) on C1 treatment after three months

Calli of *C. forskohlii* successfully generated from leaf segments on MS medium supplemented with kinetin alone or in combination with 2,4-D. Kinetin alone was the best induced maximum growth of callus (Reddy et al., 2001). Also, various growth regulators were investigated to promote calli production from *C. forskohlii*; however, the medium containing 1-2 mg/L of both NAA and BAP produced the maximum results (Yang et al., 2003). Among different growth regulators that were used, callus formation was more responsive on 2,4-D containing media (Praveena et al., 2012). Furthermore, different explants (petiole, leaf, node, internode and root) were used to induce calli from *C. forskohlii* on both media (MS and B5) fortified with different growth regulators. It was found that only the presence of 2,4-D had a positive effect (Sreedevi et al., 2013). Additionally, the friable calli of *C. forskohlii* were also observed from shoot tip explants on MS-medium containing 2.0 mg/L BAP + 1.5 mg/L 2,4-D (Vibhuti & Kumar, 2019). In our study, the 2,4 D + Kin combination more successfully induced calli from leaf explants than roots, which was the opposite of that seen with root explants, which responded to all tested treatments, including 2,4 D + Kin and NAA + BA combinations. However, the best friable calli could only be produced by using 2,4-D + Kin combination in both the leaf and root explants.

3.2 *In vitro* plantlet formation and multiplication

Table 2 and Figure 2 present the effects of BA or Kin at different concentrations (0.5, 1.0, and 2.0 mg/L) on the growth of the plantlets. According to the data in Table 2, growing explants on MS medium supplemented with 0.5 mg/L Kin recorded the maximum shoot length (4.2 cm), number of leaves (23.3), and number of shoots (3.3). From the data, it can be seen that the application of Kin was preferred compared to BA on shoot length, the number of shoots, and the number of leaves. Additionally, increasing the concentration of Kin or BA to 2.0 mg/L reduced the growth of new shoots. Rooted shoots were observed in all concentrations of BA or Kin, and the maximum percentages were recorded at 0.5 and 1.0 mg/L of cytokinin. It was also noted that increasing the concentration of cytokinin to 2.0 mg/L decreased the rooting percentage. Rooted shoots on 0.5 mg/L BA and Kin are shown in Figure 3.

Table 2. Effect of different concentrations of BA or Kin on shoot length (cm), number of leaves, number of shoots, and percentage of rooting of *Coleus forskohlii* plantlets after one month of culturing

Treatments (mg\L)		Shoot Length (cm)	No. of Leaves	No. of Shoots	Percentage of Rooting
BA	0.5	2.8 ± 0.2^{b}	14.7 ± 1.8^{b}	2.7 ± 0.3^{ab}	100%
	1.0	2.5 ± 0.3^{bc}	13.3 ± 0.7^{b}	3.3 ± 0.3^{a}	100%
	2.0	2.3 ± 0.2^{bc}	12.0 ± 1.2^{b}	2.3 ± 0.3^{ab}	82%
Kin	0.5	4.2 ± 0.3^{a}	23.3 ± 3.3^{a}	3.3 ± 0.3^{a}	100%
	1.0	4.0 ± 0.3^{a}	15.3 ± 0.7^{b}	2.7 ± 0.3^{ab}	100%
	2.0	1.8 ± 0.2^{c}	11.3 ± 0.7^{b}	1.7 ± 0.3^{b}	70%

Results are the mean±SE of at least three replicates. One-way ANOVA with least significance differences comparison tests were used at $p<0.05$.

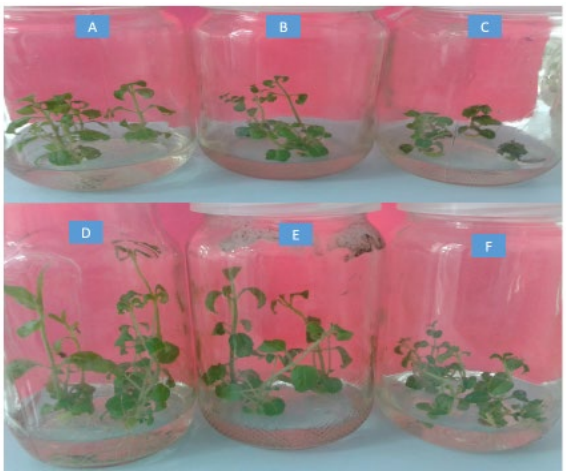

Figure 2. Plantlet formation from shoot tip explants of *Coleus forskohlii* on different concentrations of BA (0.5,1.0 and 2.0 mg/L, A, B, C) and Kin (0.5, 1.0 and 2.0 mg/L, D, E, F) after one month

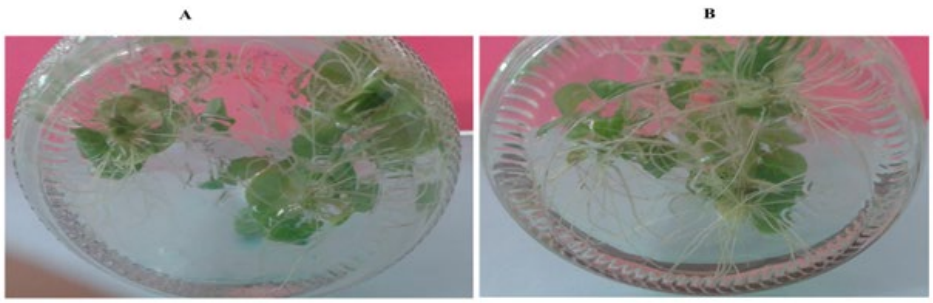

Figure 3. Root formation of *Coleus forskohlii* shoot on MS-medium containing BA (A), and Kin (B)

Our data were in accordance with those reported by Bhattacharyya & Bhattacharya (2001), who confirmed that the plantlets of *C. forskohlii* grown from stem tip explants in MS media with 0.57 mM indole-3-acetic acid and 0.46 mM kinetin grew at a rate of 12.5 shoots per explant in 35-40 days. Approximately 100% of the rooted shoots and micro-propagated plants were successfully established in soil with a good survival rate. While, in another investigation, entire *C. forskohlii* plants were grown in MS media containing 2.0 mg/L BAP via direct multiplication at a rate of 15-20 shoots per explant. All of the plants rooted, and the survival rate of fully mature plants was 100% (Kaul et al., 2015).

3.3 Phenolics and flavonoids content

Phenolics and flavonoids are significant groups of secondary components in plants synthesized by the phenylpropanoid pathway, which are known to be responsible for microbial infection attacking plants (Kelly et al., 2002). Phenolics and flavonoids also

involve in various pharmacological activities, including activities against cardiovascular diseases, cancers, age-related diseases, and also act as protective agents against bacterial and viral diseases (Middleton, 1998; Pandey, 2007; Du et al., 2011). The current study aimed to detect various phenolic and flavonoid components in 80% methanol and aqueous extracts of different cultures. The results presented in Table 3 showed 14 compounds in different extracts. The major compound was gallic acid, which displayed its maximum concentration in the 80% methanol extract of both calli from roots and leaves of 23.43 and 23.62 µg/g DW, respectively, and 37.00 µg/g DW in aqueous extract of plantlet culture. However, lower concentrations of gallic acid in the aqueous extract of both root and leaf calli were recorded at 7.56 and 10.85 µg/g DW, respectively. The second major compound was cateachin, which was detected in the aqueous extract and recorded at 13.88 and 8.31 µg/g DW in both root and leaf calli, respectively. However, it was not detected in the aqueous extract of plantlet culture. The compound chrysin was found in a significant proportion in the 80% methanol extract of leaf calli (6.65 µg/g DW), at 1.92 µg/g DW in root calli, and at 0.90 µg/g DW in plantlet culture. p-Hydroxybenzoic acid was found in the aqueous extract of root calli (1.69 µg/g DW), and syringic acid was found in the aqueous extract of leaf calli (1.19 µg/g DW). Additionally, apigenin was found in the methanol and aqueous extracts of leaf calli (1.13 and 2.98 µg/g DW, respectively). The rest of the compounds were found in small proportions of less than 1µg/g DW. The chromatogram of different standard phenolic and flavonoid compounds is shown in Figure 4.

Table 3. Phenolic and flavonoid compounds (µg/g DW) in 80% methanol and aqueous extracts of different cultures of *Coleus forskohlii*

Phenolic Compounds	**80% Methanol Extracts (µg/g DW)**			**Aqueous Extracts (µg/g DW)**		
	Callus Culture from Root	**Callus Culture from Leaf**	**Plantlet Culture**	**Callus Culture from Root**	**Callus Culture from Leaf**	**Plantlet Culture**
Gallic	23.428	23.617	18.26	7.56	10.845	37.00
Protocatechuic	0.003	0.037	ND	0.803	ND	5.74
p-hydroxybenzoic	0.072	0.172	0.016	1.689	ND	ND
Gentisic	0.013	0.043	0.112	ND	ND	ND
Catechin	0.007	ND	0.002	13.879	8.307	ND
Chlorogenic	0.375	0.407	0.345	ND	ND	ND
Caffeic	ND	0.031	0.019	ND	ND	ND
Syringic	ND	0.012	0.002	ND	1.185	0.863
Vanillic	ND	0.062	0.008	0.943	ND	0.49
p-coumaric	ND	0.093	ND	ND	ND	ND
Rutin	0.316	0.432	ND	ND	ND	ND
Apigenin-	ND	1.129	0.136	ND	2.977	ND
Kaempferol	0.020	0.037	0.027	ND	ND	ND
Chrysin	1.921	6.648	0.895	ND	ND	ND

ND (Not detected)

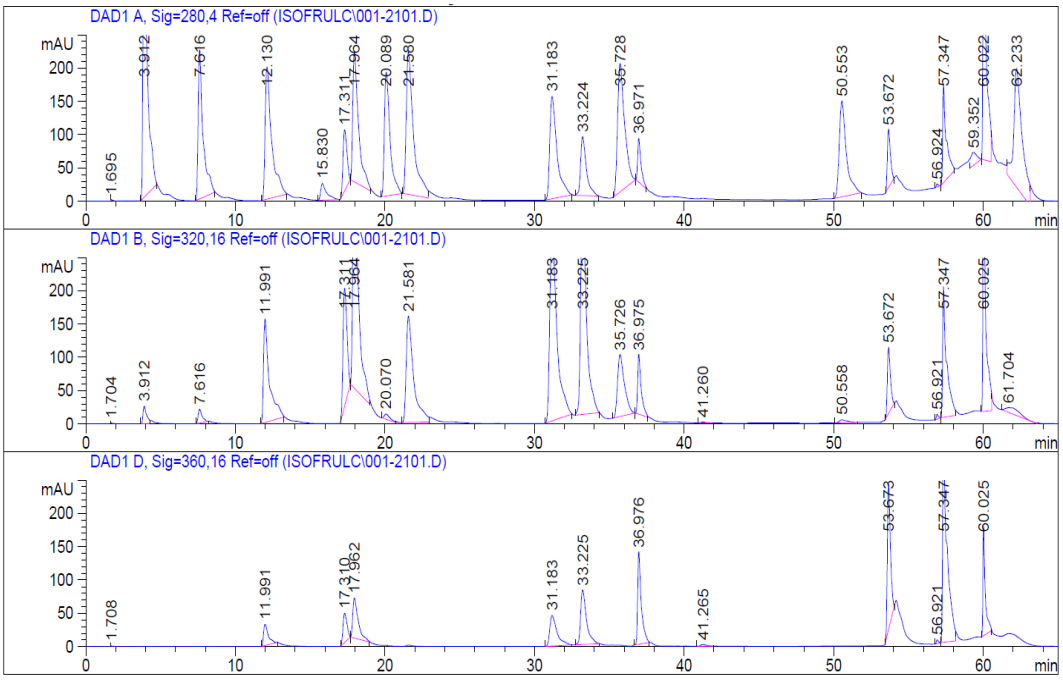

Figure 4. HPLC chromatogram of phenolic and flavonoid standard compounds showing the peaks obtained for the mixture containing all 14 compounds at: λ=280 nm (gallic acid, protocatechuic, p-hydroxybenzoic, cateachin, syringic, vanillic, *p*-coumaric, and chrysin); λ=320 nm (gentisic, chlorogenic, caffeic, and Apigenin); and λ=360 nm (rutin and kaempferol)

Our findings are in agreement with the research by Ganash and Qanash (2018), who identified gallic acid (25.42 g/g DW) as a significant component in the *C. forskohlii* plant which had been extracted with methanol and detected by HPLC.

In the current investigation, undifferentiated calli tissues yielded 23.43 and 23.62 g/g DW for root and leaf calli, respectively. In our findings, novel chemicals such catechin, apigenin, and chrysin were also found in our investigation. Ferulic acid was also identified as a prominent component in earlier work by Ganash and Qanash (2018). Total phenolic and flavonoid levels in a methanol extract of *C. forskohlii* callus culture were measured in another study, and the results showed that these levels were 1.19 and 1.14 mg/g DW, respectively (Ibrahim et al., 2018).

3.4 Gas chromatography–mass spectrometry

The primary method for obtaining chemicals with biological activity is extraction. To achieve the biological efficacy of these chemicals, solvents are crucial and specialized agents (Chang et al., 2002; Ajanal et al., 2012). To obtain compounds with antibacterial action in this investigation, two solvents (chloroform and ethyl acetate) were utilized. This required GC-MS analysis of the most significant compounds detected in the extracts. Table 4 illustrates the most abundant components in the chloroform extract from different cultures (15 compounds). Fatty acid compounds appear to be the most abundant constituents in the chloroform extract. Examples are 9,12-octadecadienoic acid, methyl ester, which was

recorded at 16.33%, 11.69%, and 25.11% in calli derived from leaf and root, and in plantlet cultures, respectively; 9-octadecenoic acid, methyl ester, recorded at 15.54%, 10.97%, and 16.98% in calli derived from root and leaf, and plantlet cultures, respectively; and hexadecanoic acid, methyl ester, which was recorded at 12.48%, 4.74%, and 27.60% in callus derived from leaf and root, and plantlet cultures, respectively. Whereas methyl stearate was only found in callus derived from leaf (4.40%). Dichloromethane was found in callus cultures from leaf and root at 2.10% and 10.44%, respectively, and 1,7-bis (3,5-bis (bromomethyl) phenyl) heptane was found at 10.66% in only callus derived from root. Other constituents of lower percentages are clarified in Table 4.

Concerning ethyl acetate extracts, the data in Table 5 shows 17 compounds detected in different cultures. 1-butanol-3-methyl acetate (isoamyl acetate) was the most abundant and remarkable compound found in both calli derived from root and leaf, and plantlet culture and recorded at 48.29%, 44.90%, and 46.06%, respectively. 1,2-benzenedicarboxylic acid, dioctyl ester was the second major compound found, being present at 15.04%, 12.15%, and 11.68% in calli derived from root and leaf, and plantlet cultures, respectively.

Fatty acid constituents like 9,12-octadecadienoic acid, methyl ester, 9-octadecenoic acid, methyl ester, and hexadecanoic acid, methyl ester were presented at lower percentages in the ethyl acetate extracts compared with chloroform extract. Other constituents were found in lower amounts in different cultures. The chromatograms and structure of the abundant compounds in chloroform and ethyl acetate extracts are shown in Figure 5. GC-MS analysis of previously published studies for *C. forskohlii* revealed some components that had different biological activities; for example, ethanol extract from root had n-hexadecanoic acid which had antibacterial and antifungal properties, botulin which had anticancer activities (Patocka, 2003), and cedrol which had anticancer effects (Breitmeier, 2006).

3.5 Antimicrobial activity

Natural compounds are increasingly being used as antimicrobials in food preservation (Omidbeygi et al., 2007). For thousands of years, medicinal herbs' antibacterial qualities have been recognized in food preservation. Bioactive chemicals in food are vital for preventing the harmful effects of free radicals as well as food deterioration caused by lipid oxidation and microbial spoilage. Among the most serious public health concerns are foodborne illnesses caused by the deterioration of food contaminated with toxins and/or disease-causing bacteria. Monitoring pathogenic microorganisms can help to decrease foodborne disease outbreaks and provide consumers with nutritious, healthy, and safe foods. Plant extracts have antibacterial activity against a variety of harmful pathogens (Luther et al., 2007; Allahghdri et al., 2010). The antibacterial activity of various extracts from *C. forskohlii* leaves and roots was studied using a variety of microorganisms. Table 6 shows the results of an investigation into the antibacterial activity of several extracts using the agar diffusion method. At 300 µg/mL, the methanol fraction of various extracts was the most effective in preventing the microbial growth of all investigated harmful bacteria. Different extracts were mediated using aqueous, chloroform, and ethyl acetate extracts. At a dose of 300 µg/mL, the zones of inhibition indicated inhibition against *C. albica* by a methanol extract of root-derived calli (25.14 mm), *B. subtilis* by a methanol extract of leaf-derived calli (16.87mm), and *S. aureus* and *C. albica* by a methanol extract of plantlet

Table 4. Chemical constituents of chloroform extracts of different cultures of *Coleus forskohlii*

No.	Compounds	Molecular Formula	% Peak Area		
			Callus Culture from Root	Callus Culture from Leaf	Plantlet Culture
1.	Dichloroacetaldehyde	C2H2Cl2O	5.55		
2.	Dichloromethane	CH2Cl2	2.10	10.44	
3.	1-Pentanol,4-amino	C5H13NO			2.66
4.	3-Pentanol, 2,4-dimethyl	C7H16O			2.55
5.	5-Methoxy-5-methyl hexanal	C8H16O2			1.38
6.	Octanal-7-methoxy 3,7-dimethyl	C11H22O2	1.19	5.26	
7.	2,4-Di-tert-butyl phenol	C14H22O		3.04	
8.	Myristic acid methyl ester	C15H30O2			1.63
9.	1,3-Dioxolane, 2-pentadecyl	C18H36O2			2.17
10.	Hexadecanoic acid,methyl ester (methyl palmitate)	C17H34O2	4.74	12.48	27.60
11.	9,12-Octadecadienoic acid, methyl ester	C19H34O2	11.69	16.33	25.11
12.	9-Octadecenoic acid, methyl ester	C19H36O2	15.54	10.97	16.98
13.	Methyl stearate	C19H38O2		4.40	
14.	methyl arachidate	C21H42O2			3.42
15.	1,7-Bis (3,5 bis (bromomethyl) phenyl) heptane	C23H28Br4	10.66		

Table 5. Chemical constituents of ethyl acetate extracts of different cultures of *Coleus forskohlii*

No.	Compounds	Molecular Formula	% Peak Area		
			Callus Culture from Root	Callus Culture from Leaf	Plantlet Culture
1.	1-Hexanol	C6H14O			1.85
2.	Hydrazine,1-methyl-1-phenyl	C7H10N2		6.07	
3.	3,5-Octadiyne	C8H10			5.68
4.	Pentane,3-ethyl-2-methyl	C8H18		3.12	
5.	1-Butanol-3-methyl acetate (isoamyl acetate)	C7H14O2	48.29	44.90	46.06
6.	Ether, butyl isopentyl	C9H20O	2.11		
7.	10-Undecenoic acid, methyl ester	C12H22O2			1.25
8.	(+)1[(Methylbenzyl) amino]cyclopropanecarboxamide	C12H16N2O	6.23		
9.	Tetradecanoic acid, 2-methyl, methyl ester	C16H32O2		1.00	
10.	Heptadecane, 2-methyl	C18H38			2.14
11.	Pentadecanoic acid, 14-methyl, methyl ester	C17H34O2			2.51
12.	9,12-Octadecadienoic acid, methyl ester	C19H34O2	3.54	3.89	4.02
13.	9-Octadecenoic acid, methyl ester	C19H36O2	3.09	3.84	5.74
14.	1-Eicosanol	C20H42O			1.28
15.	1-Heptadecanol	C21H18		1.74	
16.	1,2-Benzenedicarboxylic acid, dioctyl ester	C24H38O4	15.04	12.15	11.68
17.	Heptacosanoic acid, methyl ester	C28H56O2	1.01		

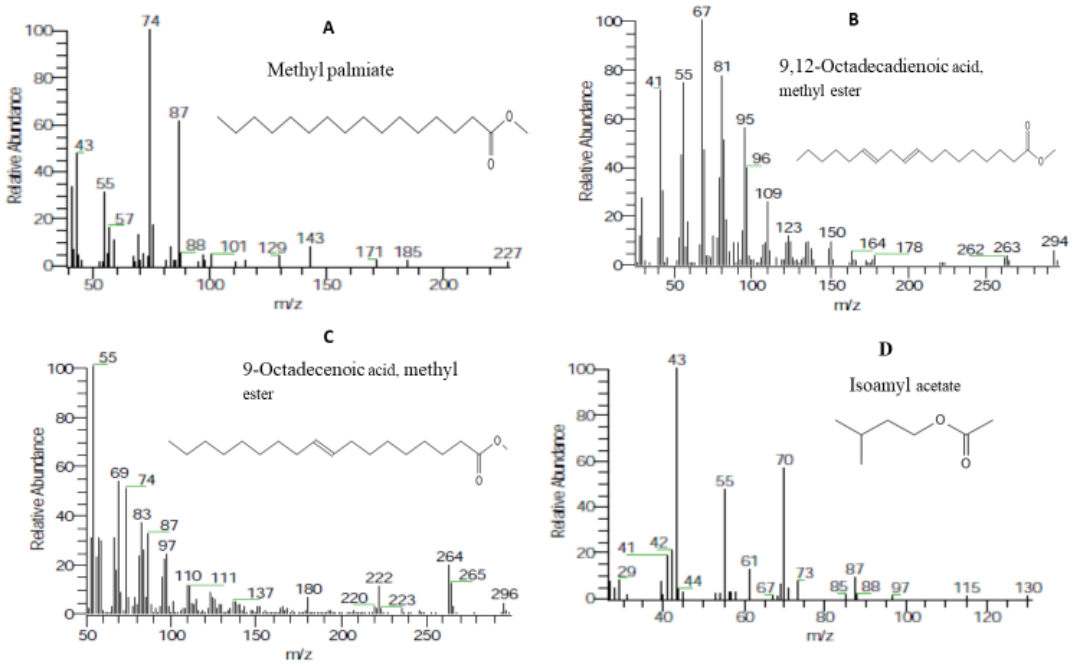

Figure 5. GC-MS chromatograms and structure of abundant compounds in chloroform extract (A, B, and C), and ethyl acetate extract (D)

(22.35 and 22.84 mm), respectively. While a 100 µg/mL of methanol extract exhibited no activity against different studied microorganisms, except the methanol extract of root-derived calli. Previous research examined the antimicrobial activity of several extracts from the stem of *C. forskohlii*, including ethanol, petroleum ether, chloroform, ethyl acetate, and n-butanol. These extracts were demonstrated for various actions against gram-positive (*S. aureus, B. cereus*), gram-negative (*E. coli, P. aeruginosa*), and *C. albicans* bacteria. According to the study's findings, the stem extract from *C. forskohlii* exhibited both biological and pharmacological properties, making it suitable for usage in both the food and pharmaceutical industries (Al-Ghamdi, 2021). The broad-spectrum antibacterial activity of *C. forskohlii* tuber was demonstrated in another study. Additionally, it was discovered that the antibacterial action was stronger against gram-negative than gram-positive bacterial strains (Khatun, 2020).

3.6 Minimum inhibitory concentrations (MIC)

Table 7 shows the results of testing the MIC effect of methanol extract on the behavior of bacteria, yeast, and fungi. Both the leaf and root calli extracts exhibited potent antibacterial action against *B. subtilis* NRRL B-94 and *E. coli* NRRL B-3703, as well as *P. aeruginosa* NRRL, *S. aureus* NRRL, *A. niger* NRRL313, *A. flavus* NRC, and *C. albicans* NRRL477. The extracts showed a broad visibility efficacy against bacteria and fungi (yeast and mold) with MIC stretching between 125 to 260 ppm.

Table 6. Antimicrobial activity of different extracts of *C. forskholii* on different tested microbes

Samples	Extracts	µg/mL	Diameter of Inhibition (mm)						
			Bacteria				Fungi		Yeast
			B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***A. niger***	***A. flavus***	***C. albicans***
Root derived calli extracts	Aqueous	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	08.76±0.47^{e}	07.65±0.45^{f}	11.00±0.87^{d}	10.46± 0.66^{fg}	09.33±47^{f}	07.56±33^{f}	09.80±45^{f}
	Methanol	100	09.16±0.47^{de}	08.35±0.37^{ef}	10.23±0.67^{d}	10.26±0.48^{fg}	08.83±0.67^{fg}	07.66±0.35^{f}	11.76±0.87^{e}
		200	15.73±0.98^{c}	14.67±0.87^{d}	15.53±0.97^{c}	15.66±0.98^{c}	14.64±0.77^{c}	13.76±0.87^{b}	15.84±0.78^{d}
		300	20.79±1.33^{a}	19.77±1.45^{b}	23.13±1.35^{a}	21.86±1.44^{a}	19.24±0.98^{a}	18.29±0.87^{a}	25.14±1.45^{a}
	Chloroform	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	07.00±0.37^{e}	08.20±0.47^{h}	00.00^{h}	00.00^{g}	11.76±0.67^{e}
		300	09.54±0.66^{de}	08.60±0.45^{ef}	10.36±0.67^{d}	11.41±0.78^{ef}	09.70±0.65^{f}	10.65±0.71^{cd}	16.82±0.97^{cd}
	Ethyl acetate	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	07.30±0.55^{e}	09.10±0.67^{gh}	08.66±0.75^{fg}	08.80±0.67^{ef}	11.00±0.87^{ef}
		300	00.00^{f}	00.00^{g}	10.96±0.85^{d}	12.43±0.97^{de}	12.60±0.87^{de}	13.15±0.71^{b}	17.81±0.98^{c}
Leaf derived calli extracts	Aqueous	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
	Methanol	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	10.16±0.67^{d}	09.78±0.47^{e}	11.12±0.71^{d}	10.38±0.67^{fg}	09.76±0.47^{f}	10.86±0.77^{c}	11.77±0.81^{e}
		300	16.87±0.91^{b}	15.73±0.87^{d}	14.33±0.77^{c}	13.96±0.67^{d}	14.25±0.65^{cd}	13.56±0.53^{b}	15.87±0.66^{d}
	Chloroform	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	00.00^{f}	00.00^{g}	10.12±0.47^{d}	09.44±0.65^{gh}	09.60±0.44^{f}	09.00±0.35^{def}	11.87±0.47^{ef}
	Ethyl acetate	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	00.00^{f}	8.66±0.33^{ef}	10.62±0.47^{d}	09.44±0.37^{gh}	09.10±0.36^{fg}	10.15±0.47^{cde}	12.22±0.67^{e}

Table 6. Antimicrobial activity of different extracts of *C. forskholii* on different tested microbes (continued)

Samples	Extracts	µg/mL	Diameter of Inhibition (mm)						
			Bacteria				Fungi		Yeast
			B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***A. niger***	***A. flavus***	***C. albicans***
Plantlet extracts	Aqueous	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
	Methanol	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	15.48±0.67^{c}	17.52±0.47^{c}	14.89±0.65^{c}	13.86±0.47^{d}	11.95±0.52^{e}	14.58±0.67^{b}	16.27±0.87^{cd}
		300	21.61±1.45^{a}	22.35±1.25^{a}	19.87±0.47^{b}	17.54±0.87^{b}	17.57±0.82^{b}	19.00±0.67^{a}	22.84±1.52^{b}
	Chlroform	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	09.72±0.47^{de}	08.66±0.31^{ef}	09.52±0.65^{d}	08.42±0.37^{h}	07.60±0.30^{g}	09.77±0.47^{cde}	10.87±0.66^{ef}
	Ethay Acetate	100	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		200	00.00^{f}	00.00^{g}	00.00^{f}	00.00^{i}	00.00^{h}	00.00^{g}	00.00^{g}
		300	00.00^{f}	00.00^{g}	07.57±0.30^{e}	07.82±0.31^{h}	08.78±0.47^{fg}	07.92±0.52^{f}	11.93±0.67^{e}

Results are the mean ± SE of at least three replicates, one-way ANOVA with least significance differences comparison tests were used at $p<0.05$.

Table 7. MIC of methanol extracts of root, leaf derived calli and plantlets of *C. forskholii* on different tested microbes

Extracts	MIC (ppm)						
	Bacteria				Fungi		Yeast
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***A. niger***	***A. flavus***	***C. albicans***
Root derived calli extract	145	138	156	160	170	175	125
Leaf derived calli extract	200	225	230	235	250	260	210
Plantlet extract	183	205	195	215	225	228	186

Our results were consistent with previous reports (Bradbury & Holloway, 1988; Furneri et al., 2002) which confirmed that the modes of action of phenolic compounds were concentration dependent. The ethnobotanical method is one of the global practices used in the selection of plants for pharmaceutical research (Cox & Balick, 1994). Although these plant extracts were reported to have antibacterial activity against seven medically important human pathogens, the bacterial killing rate has been studied to confirm this proposal on a scientific basis. Our findings suggest that *C. forskohlii* root calli extract possesses broad-spectrum antibacterial action. In the future, these extracts could be used to create a formulation to treat infectious disorders caused by the test bacteria. The findings are consistent with earlier research (Palombo & Semple, 2001; Senthilkumar et al., 2010).

Utilizing a variety of explants to produce the callus is crucial when using tissue culture techniques, which are intended to select the best conditions to obtain the highest effectiveness. Our results confirmed that the callus produced from the roots was more effective on all the microbes under study.

4. Conclusions

Finding natural sources from plants that have biological effects is of interest in a lot of studies today. *In vitro* calli cultures from medicinal plants provide these natural compounds under controlled, continuous and inexpensive conditions. In this study, calli cultures derived from two explants of *C. forskohlii* were extracted with different solvents. The obtained results showed differences in the quality and quantity of detected components, as well as the ability to inhibit various studied microbes. The methanolic extracts from different cultures were the most effective. Therefore, this study recommends the use of the methanolic extract of callus root as an antimicrobial agent. Further studies should be investigated to use it as a safe preservative agent in the food industry.

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6. Authors' Contribution

MI: conceived and designed the study, performed tissue culture techniques from *C. forskohlii*, performed different extracts and chemical analysis, performed data collection and analyzed data. MA: performed antimicrobial activity of various extracts, performed data collection and analyzed the data. AE: performed data collection and analyzed the data. HT: supervised the manuscript. All authors contributed to writing the manuscript.

7. Conflicts of Interest

The authors declare that they have no competing interests.

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