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Antimicrobial and antioxidant potentials of callus cultures of *Convolvulus microphyllus* Sieb. ex Spreng.

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ABSTRACT

Background & Aim: *Convolvulus microphyllus* Sieb. ex Spreng. (Family: Convolvulaceae) known as "Shankhpushpi" is reported to be brain tonic and useful in CNS disorder, hypertension, thyrotoxicosis and ulcer. Tissue culture technique has been optimized for the large scale production of medicinally important plant independent from climatic and geographic conditions. Antioxidant and antimicrobial activities were performed and compared with *in vivo.* Active compounds with biological activities were isolated, identified from the callus extracts. These biological constituents can be propagated in large amount with low cost use tissue culture techniques.

Experimental: Cell cultures of *C. microphyllus* have been established using Murashige and Skoog's (MS) medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2, 4-D). Callus was harvested at different time intervals of 2, 4, 6 and 8 weeks and their antimicrobial and antioxidant potentials along with the isolation of active compounds isolation were carried out using established protocols.

Results: Ethanolic extracts of 2 weeks-old callus demonstrated appreciable antifungal activity against *Penicillium chrysogenum* and *Tricophyton rubrum* (inhibition zone of 14.66 ± 0.66 and 14.00 ± 0.57 mm respectively) while maximum antibacterial activity was recorded in 6 and 4 weeks-old callus against *Klebsiella pneumoniae* (Inhibition zone of 14.66 ± 0.61 mm and 14.33 ± 0.59 mm respectively). Antioxidant potentials were more in plant extract (IC_{50} 0.055 mg/ml and 510 ± 20.02 ascorbic acid equivalents) as compared to callus. Phenolic acids viz., caffeic-, *p*- coumaric-, ferulic-, gallic-, vanillic- and syringic acids were isolated and screened for antimicrobial efficacy.

Recommended applications/industries: The callus extract shows similar results as that of *In vivo* plant. Two week old callus exhibit most profound antifungal and 4 to 6 week for antimicrobial activities. Callus extract shows similar bio-potentials and secondary metabolites level, so it can be used for large scale production of biologically active phytochemicals with antimicrobial properties.

1. Introduction

Plant-derived polyphenols are of great importance because of their potential antioxidant and antimicrobial properties (Andrade *et al*., 2015; Adamczak *et al*., 2020). Antioxidants are important in the prevention of human diseases, function as free radical scavengers, complexing agents for pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation (Albuquerque *et al*., 2021; Bouarab-Chibane *et al*., 2018).

Convolvulus microphyllus Sieb. ex Spreng. Commonly known as "Shankhpushpi" in India. Whole plant is extensively used in indigenous system of

medicine for its memory enhancing ability and provides strength to the heart and prevents thinning of blood (Singh *et al*., 1979; Shah and Bole, 1960). Ethanolic extract of the plant has smooth muscle relaxant, antiulcer (Sairam *et al*., 2001), antihyperlipidaemic (Chaturvedi *et al*., 1995), antioxidant (Parihar and Hemnani, 2003), antithyroid activities. On chemical investigation, sterols, fatty acid chains hydrocarbon, primary alcohols were reported (Bisht *et al*. 1978; Barar and Sharma, 1965; Srivastava and Despande, 1975). Its alkaloids demonstrated memory enhancing while alcoholic extract hypotensive and anticonvulsant effects (Ahmad *et al*., 2007).

In the present study, the cell cultures of *C. microphyllus* were established and bioactive phenolic acids were isolated. Level of phenolic acids, antimicrobial and antioxidant activities in undifferentiated cultures of different age groups were carried out along with their antimicrobial potentials against pathogenic microbes.

2. Materials and Methods

2.1 Plant material and culture conditions

During the course of studies, authenticated whole plants of *C. microphyllus* were collected from the campus of University of Rajasthan, Jaipur, India. Voucher specimen was compared with Herbarium specimen (Herbarium Sheet No. 20235) in the Department of Botany, University of Rajasthan, Jaipur, India.

Leaf explants from 6 months-old plant of *C. microphyllus* collected from University campus, Kota, India, young leaf explants were excised and surface-sterilized using standard procedures. Sterilized pieces (1 inch) were cultured on Murashige and Skoog medium (MS; Murashige and Skoog 1962) consisting of basal salts, vitamins, 3% (w/v) sucrose and 0.8% agar with different concentrations of growth regulators viz., 2,4-dichlorophenoxyacetic acid (2,4-D), indole acetic acid (IAA) and kinetin (Kn) on 0.5, 1, 1.5 and 2 mg/l). The pH of the medium was adjusted to 5.8 and autoclaved at 15 psi for 15 min. Cultures were maintained at 26±2^{o}C under 16 h photoperiod illuminated by fluorescent light (2000-3000 lux) and 55±5% relative humidity.

2.2 Extract preparation

For preparation of ethanolic extracts, whole plants (including leaves, roots, stem and flowers) were harvested, shade-dried and powdered. Similarly, callus at 2, 4, 6 and 8 weeks was harvested weighed, their moisture and growth indices were calculated. 100 g of plant material and 20 g of dried callus was extracted in ethanol (3×18 h), filtered through Whatman filter paper no. 1, and then filtrate evaporated to dryness *in vacuo* and stored at 4^{o}C until used.

2.3 Isolation of phenolic acids

Each of the extract was applied on silica gel G coated plates (0.4-0.5 mm) along with the standard reference compounds in air-tight chamber containing different solvent systems (Table 2). Each of the plate was exposed to I_2, NH_3 vapors, observed under UV light and sprayed with Folin-Ciocalteu's reagent. Rf values were calculated and compared with the standard purchased from Merck, Germany.

Based on their TLC behavior and IR spectra (KBr pellets on A 400S Shimadzu FTIR spectrometer) compounds were identified. Melting point was measured using Toshniwal Melting Point apparatus.

2.4 Total phenolics and flavonoids contents

Phenolics content were calculated spectrophotometrically using Folin-Ciocalteau method (Wu *et al*., 2004) at 750 nm (Pharmaspec UV- Vis spectrophotometer by Shimadzu). A standard calibration curve of gallic acid in methanol (1-50 mg/mL) was prepared and total phenolics expressed in mg of gallic acid equivalents (mg GAE/g dw; Bray and Thorpe 1954). Total flavonoids estimated by $AlCl_3$ spectrophotometic method (Zhishen *et al*., 1999) by preparing standard curve of quercetin (1-10 mg/mL). The total flavonoids were expressed as mg of quercetin equivalents (mg QE/g dw) of extract and statistically analyzed.

2.5 Quantification of isolated compounds

Quantification was carried out spectrophotometically using preparative TLC on activated silica gel G plates (Wu *et al*., 2004). 100 mg of methanolic extract was applied on TLC plates, developed in selective solvent systems, air-dried and visualized in I_2 vapors. The spots

were marked and collected in separate test tubes. Five mL of spectroscopic methanol was added, shaken vigorously, centrifuged and the supernatant was collected separately. 100 µl of the concentrated compound was mixed with 2.5 ml of deionized water, followed by the addition of 0.1 ml (2N) Folin-Ciocalteu reagent and allowed to stand for 6 min. Later, 0.5 ml of 20% sodium carbonate solution was added, incubated (30 min) and absorbance was taken at 760 nm. Similarly, standard curves of phenolic acids were prepared using 1000-62.5 µg/mL concentrations and compared with isolates to their respective standard regression curve (mg/g dw).

2.6 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical activity

This activity was determined using method of Fogliano *et al.*, (1999). For quantitative assay, each of the extract (0.008 g) was dissolved separately in methanol and various concentrations were prepared. Each of the 2.5 ml of test extracts was mixed with DPPH (0.002 g 10 ml/l methanol) and allowed 30 min for reaction. Optical density (OD) was measured at 517 nm using a UV-Vis spectrophotometer. The IC50 value expresses the amount of extract necessary to decrease the absorbance of DPPH by 50%. This value was determined graphically by plotting the absorbance against the extract concentration and calculated from the linear regression curve of DPPH. Percent inhibition of DPPH was calculated by following equation:

$$\% \text{ Inhibition} = 1-(OD_{Sample}/OD_{Control}) \times 100$$

Where, OD_{Sample} is the absorbance of the test sample and OD_{Contol} as the absorbance of the test control.

2.7 Ferric ion reducing antioxidant potentials

Total reducing power of extracts was determined spectrophotometrically (Yen and Chen, 1995). OD was measured at 700 nm. Ascorbic acid *was* used as positive control by preparing a standard curve (1-10 mg/ml), the absorbance of extracts were compared with data of ascorbic acid and results were expressed as ascorbic acid equivalent AAE mg/l dry weight.

2.8 Assay of antimicrobial activity

For antimicrobial screening, pure cultures of test bacteria, *Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Raoultella planticola* and *Staphylococcus aureus* and test fungi *Candida albicans*, *Penicillium chrysogenum* and *Tricophyton rubrum* obtained from Institute of Microbial Technology, Chandigarh, India while *Aspergillus flavus* and *A. niger* obtained from Indian Agriculture Research Institute, New Delhi, India were used. Antimicrobial assay was performed by agar well diffusion method (Boyanova *et al*., 2005). Wells of 6 mm diameter were cut on sterile nutrient and potato dextrose agar plates and swabbed with an overnight broth culture of the organism. 40 µl (4 mg) of the extracts were filled into each of the wells and incubated at 37±0.2°C. Antibacterial activity in terms of zones of inhibition (mm) recorded after 24 hrs of incubation. All experiments performed in triplicate. Gentamycin (10 mcg/well) for bacteria and ketonocozole (10 mcg/well) for fungi were used as positive controls.

In the present experiment, for antimicrobial potentials inhibition zones of extracts and minimal inhibition concentration (MIC) values of the isolated compounds were compared. Similar method was used for the determination of MIC of isolated pure compounds. Serial dilutions of the extracts ranging 2000 µg to 62.5 µg were prepared and administered in previously inoculated plates. Both experiments were carried out in triplicate and statistically analyzed.

2.9. Statistical Analysis

Statistical analysis was carried out by One-way analysis of variance (ANOVA) test using a statistical package program (SPSS 10.0) and the significance of the difference between means was determined by Duncan's multiple range test at (P<0.05) significant level. Analysis was carried out in triplicate and mean±SD of three parallel measurements.

3. Results and discussion

3.1. Callus induction

Leaf callus of *C. microphyllus* has been established using 2,4-D, Kn and IAA individual and/or in combination. Among the concentrations used, 0.5 mg/l 2,4-D gave better callus induction (80% response). 2,4-D in combination with IAA (0.5 mg/l each) also proved useful in the callus induction (80%). However, in the present study kn could not give appreciable response.

Colour and texture of the callus at different hormones combinations have been documented (Table 1).

Table 1. Callus induction from leaf explants on different combinations of 2, 4-D, IAA and kinetin.

Hormone concentration (mg/l)			Explant response	Callus growth	Colour	Texture
2, 4-D	Kinetin	IAA				
0.5	-	-	80	++++	LBN	FR
1	-	-	60	++	LBN	FR
1.5	-	-	60	++	LBN	FR
2	-	-	40	++	LBN	FR
0.5	0.5	-	30	+	BG	CM
1	1	-	20	+	BG	CM
1.5	1.5	-	20	+	BG	CM
2	2	-	-	-	BG	CM
0.5	-	0.5	80	+++	GN	FR
1	-	1	60	++	GN	FR
1.5	-	1.5	60	++	GN	FR
2	-	2	20	+	GN	FR

Evaluation made 8 week after culture initiation. Results are shown in %. Experiment contained three replicates per treatment.
+, Low; ++, moderate; +++, intense; LBN, light brown; BG, brownish green; GN, green; FR, friable; CM, compact.

For further experiments, callus was multiplied on MS medium containing 2,4-D 0.5 mg/l. In several studies plant cell cultures have been used for large scale production biologically active constituents used for cosmetics and food industries (Eibl *et al.*, 2018). In this approach *Convolvulus* species, has been previously explored for cell culture studies (Zafar *et al.*, 2005). In our experimental cell culture profiling both 2,4-D and IAA favors callus induction and biomass production.

3.2. Qualitative and quantitative analysis of phenolic acids

On TLC, 6 phenolic acids namely caffeic-, *p*-coumaric-, ferulic-, gallic-, vanillic- and syringic acids were detected and identified both *in vivo* plant and in callus (Table 2). On quantification, levels of *p*-coumaric acid was higher in 2 weeks-old callus (380.33 ± 0.88 µg/g dw), whereas vanillic acid was maximum in 6 weeks-old callus (401.66 ± 1.15 µg/g dw). Higher levels of phenolic acids viz. caffeic, gallic and ferulic acids were recorded in 8 weeks-old callus (514.66 ± 0.89, 502.66 ± 1.76, 205.66 ± 0.66 µg/g dw, respectively) as compared to others, whereas levels of syringic acid remained same in all stages of callus (Table 2). On comparing the levels of gallic acid, syringic acid was higher in the *in vivo* plant than the callus (575.00 ± 1.15 and 365.66 ± 1.85 µg/g dw, respectively).

Table 2. Chromatographic and spectroscopic data of the isolated phenolic acids from *C. microphyllus in vivo* and *in vitro*.

Isolated compounds	R_f in solvent systems*						m.p. (^{0}C)	IR (ν_{amx}) cm-1(KBr)	H^1 NMR Spectra (δ)
	I	II	III	IV	V	VI			
Caffeic acid	-	-	-	-	-	0.96	195-198	3440, 3220-2480, 1640, 1530, 1520, 1448, 1212, 1172, 1118, 972, 899, 850, 812	11.92 (1H , s, COOH), 8.29, (1H, br, s, OH), 8.07 (1H, br s, OH), 7.52 (1H, d, J=15.9 Hz, H-7), 7.08 (1H, s, H-2), 6.91 (1H, d, J=8.1 Hz, H-5), 6.83 (1H, d, J=8.1 Hz, H-6).
Ferulic acid	0.73	0.53	0.53	0.52	-	-	168-171	3435, 2320-3120, 1685, 1620, 1516, 1432, 1320, 1270, 1205, 1110, 1040, 930, 845, 805	12.21 (1H, s, COOH, 1H), 9.08 (1H, s, OH), 7.59 (1H, d, J=15.9 Hz, H-7), 7.36 (1H, s, H-2), 7.04 (1H, d, J=8.1 Hz, H-5), 6.93 (1H, d, J=8 Hz, H-6), 6.32 (1H, d, J=15.9 Hz, H-8), 3.93 (3H, s, OCH_3).
Gallic acid	-	-	-	-	0.46	-	250-252	3520-3260, 2650-2960, 1700, 1618, 1540, 1440, 1246, 1080, 863	9.21 (1H, s, COOH), 6.96 (2H, s, H-2, 6), 3.86-3.37 (4H, br, OH).
p- Coumaric acid	0.67	0.55	0.04	0.45	-	-	210-213	3380, 3330-2920, 1672, 1627, 1602, 1512, 1450, 1290, 1245, 1215, 1170, 997, 830	12.09(1H, s, COOH, 1H), 9.18 (1H, s, OH), 7.59(1H, d, J=16.2 Hz, H-7), 7.00 (2H, d, J=7.8 Hz, H-2, 6), 6.86 (2 H, d, J=7.8 Hz, H-3, 5), 6.29 (1H, d, J=16.2 Hz, H8).
Syringic acid	0.72	0.16	0.60	0.67	-	-	206-209	3370, 1690, 1615, 1520, 1450, 1382, 1310, 1245, 1210, 1110, 850, 763	12.32 (1H, s, COOH), 9.68 (1H, s, OH), 7.20 (2H, s, H-2, 6), 3.80 (3H, s,-OCH_3).
Vanillic acid	0.71	0.55	0.60	0.50	-	-	210-213	3484, 3060-2480, 1680, 1600, 1518, 1434, 1284, 1240, 1210, 1105, 1028, 910, 760	12.48 (1H, s, COOH), 9.85 (1H, s, OH), 7.45 (1H, d, J=8.7 Hz, H-5), 7.43 (1H, s, 2H), 3.80 (3H, s, OCH_3).

*Solvent systems: I- Methanol-acetic acid-water (18 : 1: 1); II- Methanol-water (4 : 1); III- Isopropanol-water (3 : 2); IV- Ethanol-water (3 : 2); V- Water-acetic acid (9 : 1); VI- Ethyl acetate-toluene-acetic acid (5 : 4 :2).

On comparison of total phenolic and flavonoids levels, it was higher in *in vivo* plant as compared to callus extract (20.73 ± 5.36 GAE/g dw and 10.65 ± 0.00 QE/g dw; Table 3). Production of phenolic acids in callus has been reported in several plants such as *Rubus chamaemorus*, *Rubus saxatilis*, *Vaccinium vitis-idaea*, *Rubus arcticus*, *Fragaria ananassa* (Rischer *et al.*, 2022). In *C. microphyllus* quantification, 3 out of 6 isolated phenolic acids were investigated higher in 8 weeks-old callus whereas *p*-coumaric acid levels was more in 2 weeks-old callus that reduces with the age.

Table 3. Quantification of phenolic acids in plant leaf callus and *in vivo* plant.

Isolated phenolic acids	Concentration of isolated compounds (µg g^{-1} dw)*				*In vivo* plant
	Age of callus in weeks				
	2	4	6	8	
Caffeic acid	281.33 ± 0.88^{a}	294.66 ± 0.88^{b}	301.33 ± 0.97^{a}	514.66 ± 0.89^{a}	365.33 ± 1.85^{a}
p-Coumaric acid	380.33 ± 0.88^{b}	360.66 ± 1.18^{b}	271.00 ± 1.15^{b}	265.00 ± 1.15^{b}	185.33 ± 0.32^{a}
Gallic acid	444.33 ± 1.20^{a}	445.66 ± 0.26^{b}	451.00 ± 1.15^{b}	502.66 ± 1.76^{b}	575.00 ± 1.15^{b}
Ferulic acid	180.00 ± 1.15^{c}	196.66 ± 0.88^{a}	204.00 ± 1.15^{c}	205.66 ± 0.66^{c}	165.00 ± 1.85^{b}
Syringic acid	319.66 ± 0.88^{a}	319.00 ± 1.15^{a}	319.33 ± 0.66^{a}	319.66 ± 0.88^{c}	365.66 ± 1.85^{b}
Vanillic acid	242.33 ± 1.45^{a}	284.33 ± 1.20^{c}	401.66 ± 1.15^{a}	302.00 ± 1.15^{c}	200.66 ± 1.76^{c}

*Data were statistically analyzed and mean values were representing mean ± standard deviation of 3 replicates per experiments. Different letters in the same line represent statistically different results according to the Duncan's multiple range test (P<0.05).

3.3. Antioxidant potentials

In DPPH radical scavenging activity, *in vivo* plant displayed better antioxidant potentials with 0.055 mg/ml of IC_{50} value as compared to callus. On comparing with different age groups of callus, 8 weeks-old callus exhibited better activity (IC_{50} value 0.075 mg/ml with % inhibition of 93.78) whereas 4 and 6 weeks-old callus demonstrated similar IC_{50} value (0.07 mg/ml, % inhibition of 74.18 and 75.64, respectively) (Table 4). These values are in harmony with the total levels of phenolics content in both the plant and callus.

In FRAP method, 510.00 ± 00 AAE/g dw antioxidant activity was demonstrated by whole plant extract followed by 8 weeks-old callus (353.33 ± 12.03 AAE/g dw) (Table 4).

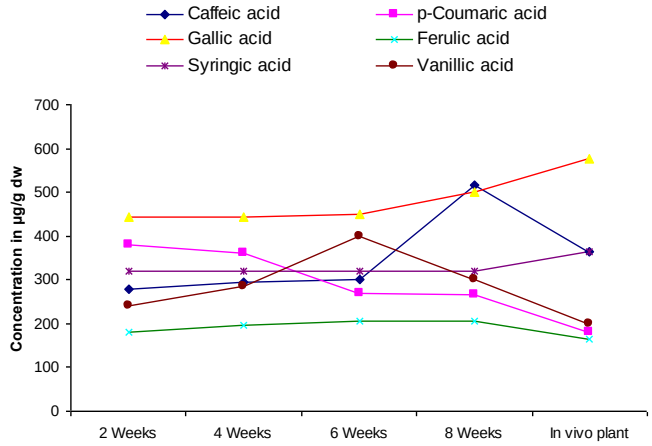

Figure 1. Levels of phenolic acids in different growth stages of callus.

Table 4. Total phenolics and antioxidant assay by DPPH and FRAP method.

Nature of extracts	Total phenolics (GAE g^{-1}dw)	Total flavonoids (QE g^{-1}dw)	% Inhibition (DPPH method) (concentration in mg ml^{-1})						Antioxidant activity in AAE mg^{-1} dw (concentration in µg ml^{-1})				
			IC_{50}	0.1	0.2	0.4	0.6	0.8	62.5	125	250	500	1000
A.2wks			0.08	59.85	63.35	67.44	69.05	71.30	30.00±0.00	31.66±1.66	50.00±0.00	90.66±1.72	255.00±15.01
4wks	17.90±12.59	5.06±0.15	0.07	67.46	69.43	71.47	71.70	74.18	30.00±0.00	41.66±3.33	53.33±1.66	131.66±3.33	288.33±4.67
6 wks	19.00±12.26	7.73±0.64	0.07	69.48	71.02	72.32	74.69	75.64	33.33±1.66	43.33±1.66	56.66±0.33	128.33±1.66	331.66±1.85
8 wks	20.36±6.67	8.62±0.03	0.075	77.38	81.75	87.80	88.39	93.78	40.00±0.00	45.00±0.00	58.00±3.00	140.00±5.78	353.33±12.03
B.	20.73±5.36	10.65±0.00	0.055	83.89	86.32	91.31	92.30	94.40	55.00±5.78	78.33±4.40	127.33±1.33	346.66±1.49	510.00±20.02
C.	-	-	0.04	62.42	80.58	93.38	93.82	94.71	-	-	-	-	-
D.	-	-	-	-	-	-	-	-	62.5	125	250	500	1000

% Inhibition = 1- (Absorbance of the sample/Absorbance of the control) × 100. A. Cell cultures (age in weeks); B. *In vivo* plant; C. Quercetin; D. Ascorbic acid. Values are expressed as means ± standard deviation (n = 3).

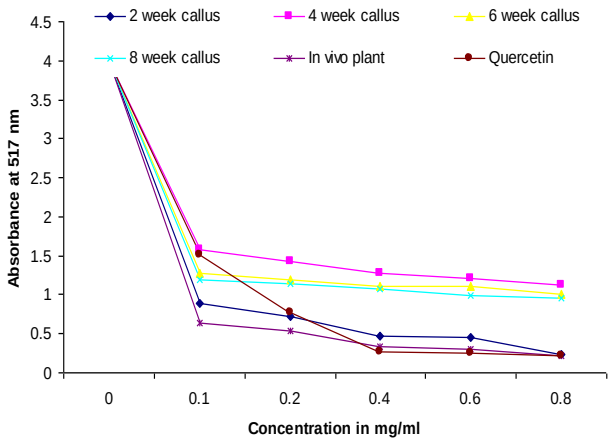

Figure 2. Antioxidant activity by DPPH radical scavenging activity in different growth stages of leaf callus with comparison to field grown plant. Data are presented as mean ± SE of each of three replicates (n=3).

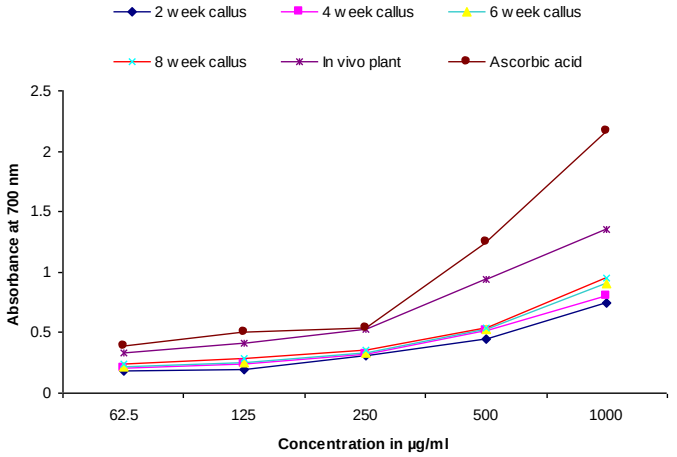

Figure 3. Antioxidant activity by FRAP reducing power in different growth stages of leaf callus with comparison to field grown plant. Data are presented as mean ± SE of each of three replicates (n=3).

2, 4 and 6 weeks-old age callus demonstrated potentials of 255.00 ± 15.01, 288.33 ± 4.67 and 331.66 ± 1.85 AAE/g dw, respectively. DPPH assay measures the free radical scavenging capacity of compounds. DPPH molecules containing stable free radical, in the presence of an antioxidant, its purple color changed to yellow on absorbency of 517 nm y (Margraf *et al*., 2015). In the FRAP assay, antioxidants in the sample reduce Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{+2}) that can be monitored by measuring the formation of Prussian blue at 700 nm. The increase in absorbance is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample (Benzie *et al*., 1999). Due to presence of higher phenolic and flavonoids contents better antioxidant potentials in both, *in vivo* and callus, was displayed. On comparison in callus, 8 weeks- and 6 weeks-old callus demonstrated higher antioxidant potentials. However, more levels of phenolic acids were found with the increased age of callus.

3.4. Antimicrobial potentials

Ethanolic extract of the whole plant did not show any appreciable activity against major Gram -ve bacteria. However, leaf callus demonstrated effective activity than the whole plant extract, where 8 weeks-old callus proved greater activity against most of the test microbes (IZ 14.00 ± 0.57, 13.66 ± 0.66 and 12.00 ± 0.57 mm against *E. aerogenes*, *S. aureus* and *C. Albicans*, respectively) (Table 5).

Table 5. Antimicrobial activity of *C. microphyllus* callus and *in vivo* plant extracts.

Microorganisms	MTCC No.		Nature of extract						
			[+]Callus extracts (age in weeks)				Whole plant extracts		
			2	4	6	8	4 mg	8 mg	10 mg
A. Bacteria									
B. subtilis	441	IZ[a]	9.33 ± 0.86	11.00 ± 0.00	11.33 ± 0.88	12.66 ± 0.66	10.00 ± 0.57	11.00 ± 0.57	15.33 ± 0.66
		AI[b]	0.38	0.45	0.47	0.57	0.41	0.45	0.62
E. aerogenes	111	IZ	9.33 ± 0.66	10.66 ± 0.66	13.33 ± 0.87	14.00 ± 0.57	10.00 ± 0.00	8.66 ± 0.32	11.66 ± 0.66
		AI	0.54	0.62	0.78	0.82	0.55	0.47	0.64
E. coli	443	IZ	-	-	-	-	-	12.33 ± 0.88	14.33 ± 0.66
		AI	-	-	-	-	-	0.68	0.79
K. pneumoniae	109	IZ	13.00 ± 0.57	14.33 ± 0.34	14.66 ± 0.66	11.33 ± 0.97	10.33 ± 0.32	10.24 ± 2.89	13.00 ± 0.57
		AI	0.54	0.59	0.61	0.47	0.43	0.41	0.54
P. aeruginosa	741	IZ	-	-	-	-	-	11.33 ± 0.97	13.66 ± 0.45
		AI	-	-	-	-	-	0.5	0.54
R. planticola	530	IZ	-	-	-	-	-	10.66 ± 0.66	12.00 ± 0.57
		AI	-	-	-	-	-	0.55	0.66
S. aureus	740	IZ	11.66 ± 0.32	12.66 ± 0.32	13.33 ± 0.88	13.66 ± 0.66	9.33 ± 0.66	14.66 ± 1.07	16.66 ± 0.45
		AI	0.48	0.52	0.55	0.56	0.38	0.58	0.66
B. Fungi									
A. flavus	16870	IZ	-	-	-	-	-	-	-
		AI	-	-	-	-	-	-	-
A. niger	322	IZ	-	-	-	-	-	-	-
		AI	-	-	-	-	-	-	-
C. albicans	4718	IZ	8.66 ± 0.66	9.66 ± 0.88	11.33 ±1.33	12.00 ± 0.57	12.33 ± 0.88	15.33 ± 0.88	17.00 ± 0.00
		AI	0.36	0.40	0.47	0.50	0.51	0.62	0.70
P. chrysogenum	5476	IZ	14.66 ± 0.66	12.33 ± 0.66	11.66 ± 0.66	10.00 ± 0.00	8.00 ± 0.00	-	-
		AI	0.66	0.56	0.53	0.45	0.36	-	-
T. rubrum	2327	IZ	14.00 ± 0.57	12.33 ± 0.86	8.00 ± 1.00	-	10.25 ± 0.30	13.66 ± 1.19	15.33 ± 0.88
		AI	0.66	0.58	0.38	-	0.48	0.65	0.73

[+]Test samples 4 mg $well^{-1}$. Standard test drugs: gentamycin for bacteria, ketonocozole for fungi (10 mcg $disc^{-1}$). [a]IZ=Inhibition zone (in mm) including the diameter of well (6 mm). AI[b] = Activity index = Inhibition zone of the sample/Inhibition zone of the standard. Values are expressed as means ± standard deviation (n = 3).

In case of *P. chrysogenum* and *T. rubrum*, 2 weeks-old callus showed greater activity as compared to others (IZ 14.66 ± 0.66 and 14.00 ± 0.57 mm, respectively).

As phenolic acids were known for its antioxidant potentials **(**Rice-Evans *et al*., 1995**)** and hence, therefore further investigated for antimicrobial properties only vanillic acid had more antimicrobial potentials as compared to other phenolic acids with MIC of 62.5 µg/ml in case of *C. albicans*, *T. rubrum* and *K. pneumoniae* (Table 6).

Table 6. MIC+ of isolated compounds of *C. microphyllus*

Isolated phenolic acid	Concentration of isolated compounds in (µg mL^{-1})							
	E. aerogenes	*K. pneumoniae*	*P. aeruginosa*	*R. planticola*	*A. flavus*	*A. niger*	*C. albicans*	*T. rubrum*
Caffeic	500	31.25	1000	250	500	-*	-	125
Ferulic	250	62.5	250	125	500	250	62.5	62.5
Gallic	500	125	500	250	-	-	250	250
p- Coumaric	250	-	1000	250	250	-	125	62.5
Syringic	1000	125	-	500	-	-	500	1000
Vanillic	250	62.5	1000	250	500	1000	62.5	62.5

+MIC = Minimum inhibitory concentration; *- No activity. Values are expressed as means ± standard deviation (n = 3).

Phenolic acids were more active against *K. pneumonia* than any other test microbes having MIC of 31.25 µg/ml in case of ferulic, caffeic acid and 62.5 µg/ml against vanillic acid. Phenolic acids demonstrated antimicrobial activity against several strains. These compounds were able to inhibit enzymatic activities of microbes (Oulahal and Degraeve 2022; Bouarab-Chibane *et al*., 2018). The site(s) and numbers of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms and the increase in hydroxylation which further results in increase in toxicity, denaturing proteins and disrupting cell membranes. Antibacterial activity of polyphenolic fraction of Kombucha against Vibrio cholerae has been previously reported (Bhattacharya *et al*., 2018). Undissociated forms of phenolic acids, prevailing at pH values below their pKa values, are uncharged and can thus cross the phospholipid bilayer of bacterial membranes and decrease intracellular pH (Wen *et al*., 2003; Pernin *et al*., 2019). Reported consequences of phenolics penetration in the cytoplasm of microorganisms encompass interruption of DNA, RNA, protein synthesis or functions, interference with intermediary metabolism [namely energy (ATP)-generating system], coagulation of cytoplasmic constituents resulting from its acidification (Mora-Pale *et al*. 2015) . In the present study 8 weeks-old callus exhibited better antimicrobial potentials than whole plant extract. Interestingly, plant and callus extracts were effective against fungi as compared to bacterial strains where maximum inhibition was demonstrated against *P. chrysogenum* and *T. rubrum*. As phenolic acids was well known for their antioxidant potentials, the isolated compounds were also tested for antimicrobial potentials, where vanillic acid followed by ferulic acid proved more effective against test microbes (Pereira *et al*.2006; Phin *et al*., 2009).

4. Conclusion

Antimicrobial activity of methanolic extracts of field grown *C. microphyllus* and callus of different growth stages were evaluated for their antioxidant and antimicrobial potentials along with isolation of active phenolic compounds. These phenolic acids are known for their antimicrobial, antiviral, antioxidant effects, which make them valuable product for the pharmacological, food (as additive), and also an efficient natural antioxidant. These compounds are able to inhibit the enzymatic activity of *B. subtilis, S. aureus, C. albicans* and *T. rubrum*. In this research paper tissue culture protocol have been generated for the large scale production of callus. This study will help for the production of biologically active ingredient of this indigenous plant at low cost for the applications in medical, pharmacology and toxicological purposes.

5. References

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