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CALLUS INDUCTION FROM THE FRONDS OF GREATER DUCKWEED (*Spirodela polyrhiza* (L.) Schleid) AND ASSESSMENT OF ITS ANTIMICROBIAL EFFICACY

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ABSTRACT

Greater duckweed (*Spirodela polyrrhiza* (L.) Schleid. are known to propagate excessively in stagnant eutrophic water bodies resulting in the contamination of daughter fronds which can affect their secondary metabolite profile. The present study was aimed in establish organogenic callus from the fronds of greater duckweeds; to optimize a protocol by utilizing different plant growth regulators for future use and to screen the antimicrobially potent metabolites present in callus. The induction of callus was successful using sterile fronds of *Spirodela polyrrhiza* (L.) Schleid as explants in MS, B5 and SH media fortified with plant growth regulators namely IBA and BAP. SH basal medium fortified with IBA and BA in 2:1 ratio was most effective in callus induction. B5 medium fortified with 2.0 µM IBA + 4.0 µM BAP showed highest callus induction in *S. polyrrhiza* with lower days to callus induction as reflective in their overall biomass. The callus extract showed efficient antimicrobial effect on *Pseudomonas aeroginosa* (MTCC accession No. 3451) with ampicillin and chloramphenicol as reference antibiotics. Presence of flavonoids like orientin, genistin and apigetrin were indicated in the LC-MS data which might be responsible for antimicrobial activity in a synergistic manner.

Keywords: Antimicrobial, callus induction, flavonoids, plant growth regulators, zone of inhibition

INTRODUCTION

Duckweeds are fast growing, free floating, minute monocotyledonous plants abundantly present in the surface of nutrient rich water bodies of tropical parts of the world (Sil and Gupta, 2021). These plants belong to the family Lemnaceae, order Alismatelis which is further subdivided into two sub-families namely Lemnoideae and Wollfiodeae (Tippery *et al*., 2015). Duckweeds are represented by 5 genera (*Lemna, Wolffia, Spirodela*, *Wolfiella* and *Landoltia* sp*)* consisting of 37 species (Bog *et al.*, 2010). The diploid chromosome number of duckweeds vary from 2n = 20 to 2n = 126 (Landolt *et al*., 1986) which is mainly attributed to the formation of asexual clones through vegetative propagation (Hastwell *et al*., 2008). Duckweeds are known to show epigenetic modifications prominently (Cao *et al*., 2015), prefer C_3 mode of carbon fixation and are known to double their biomass in 48-72 h (Driever *et al*., 2005). Generally warm and sunny conditions are best suited for the growth of duckweeds (Goppy and Murray, 2003). Duckweeds can store phosphate and nitrate within their vacuoles in the form of oligo-cyclic and high-molecular compounds (Khondkar *et al*., 1994) and are able survive under modified conditions by rapidly adapting to the changed environment (Ghosh, 2005).

Spirodela polyrrhiza (L.) Schleid. also known as greater duckweed or giant duckweed, is predominantly cosmopolitan in distribution with remarkable degrees of structural reduction exhibited by its fronds (Kim and Kim, 2000). It reproduces mainly through vegetative propagation where daughter fronds remain attached to the mother fronds with the help of connective stalk. *S. polyrrhiza* is known to produce hibernating structures called turions, which on return of favourable conditions develops into new plants (Landolt, 1986). Turion formation is enhanced under deficiency of phosphate in water bodies (Schwalbe, 1999). The lower epidermis of duckweed species like *Lemna minor* and *L. gibba* are often found to be infested with cyanobacterial colonies like *Gloeotrichia* sp., *Nostoc* sp., *Cylindrospermum* sp., *Calothrix* sp. and *Anabaena* sp. (Coler and Gunner 1969, Zuberer, 1982) that thrive on the organic debris released from duckweeds creating a microenvironment favouring their growth (Gupta and Sil, 2023). *S. polyrrhiza* unfolds immense potential for biotechnological applications, including bioremediation, biofuel production, and pharmaceutical compound synthesis. However, the limited availability of efficient *in vitro* regeneration protocols hinders the exploitation of its full potential. Callus induction is a critical step in plant tissue culture, enabling mass propagation, genetic engineering, and secondary metabolite production. An important factor that controls *in vitro* growth of duckweeds is hydrogen ion concentration of medium (Clark, 1925, Hicks and Lawrence, 1930). Despite its importance, the callus induction in *S. polyrrhiza* is still understudied.

Giant duckweeds contain a plenty of secondary metabolites which show anti-oxidative, anti-microbial and anti-cancerous properties. The concentration of such metabolites vary when grown under *in vitro* conditions or in callus. *S. polyrrhiza* contains high quantity phenols and flavonoids that are extremely important for their survival in aquatic ecosystems. Like other duckweeds, *S. polyrrhiza* are rich source in proteins that contain essential amino acids in sufficient proportions and can supplement the growth of poultry birds and fishes as protein source because of its amino acid profile. The fronds store sufficient amount of beta-carotene and lycopene, whose concentrations increase drastically when cultured in nutrient rich medium. The antimicrobial potentials of *S. polyrrhiza* are very few or scanty, however reports reveal that the ethanolic and methanolic extracts of *S. polyrrhiza* contain various flavonoid compounds (Qiao *et al*., 2011). The present experiment was aimed to optimize callus induction in *S. polyrrhiza* using various plant growth regulators and culture conditions, providing a foundation for future biotechnological applications. By establishing an efficient callus induction protocol, we can unlock the full potential of this aquatic plant, contributing to the development of sustainable solutions for microbial drug resistance and other threats imposed by pathogenic microbes.

MATERIALS AND METHODS

Callus induction

Fronds of *Spirodela polyrrhiza* (identified by Botanical Survey of India, CNH, Shibpur, Kolkata, India) were collected from pure liquid culture in tissue culture laboratory of the Department of Botany, The University of Gour Banga, Bengal (India) maintained at pH 5.8, photoperiod 16:8, light intensity 22.2 µmol m^{-2} s^{-1} and temperature 28±2 °C in SH basal medium (HiMedia PT059) and selected as explants. The fronds were collected in mid log phase and surface sterilized by 0.1% mercuric chloride for 1 min. The explants were then transferred to three different types of basal culture media namely Murashige and Skoog basal medium (Murashige and Skoog,1962) HiMedia, product code-PT021; Gamborg B5 basal medium (Gamborg *et al.*,1968) HiMedia, product code-PT016; and Schenk & Hildebrand basal medium (Schenk and Hildebrandt, 1972) HiMedia, product code-PT138 along with three plant growth regulators (PGRs) in combination namely indole butyric acid (IBA, HiMedia, product code-PCT0804); 2,4-dichlorophenoxy acetic acid (2,4-D, HiMedia, product code-PCT0825) and 6-benzyl aminopurine (BAP, HiMdedia, product code-PCT0802). 2,4-D was supplemented at the concentration of 1.0 µM to MS basal and Gamborg B5 basal media. The three basal media (MS, SH, B5) were supplemented with two combinations of IBA *viz.*, 1.0 µM and 2.0 µM, separately. BAP was added to the tissue culture media in double concentration of IBA (2 µM and 4 µM) separately. The three basal culture media selected i.e. MS, SH and B5 were treated as control which did not receive any supplementation of PGRs. The pH of culture media was maintained at 5.8-5.9. The entire set up of each combination mentioned earlier including control were set up in three replicates with 10 culture tubes in each replicate. For first three days, all culture tubes containing explants of *S. polyrrhiza* were kept in complete darkness. Then these tubes were maintained at 24 ± 2°C, 80% relative humidity, photoperiod of 16:8 h and 2200 Lux light intensity. Days to callus induction (DCI) were observed for each separate concentrations and percentage of callus induction (%CI) were calculated.

Preparation of callus extract

One gram callus *S. polyrrhiza* was grinded in a mortar pestle with 20 mL aqueous methanol (HPLC grade FINAR). The mixture was kept in a conical flask for 48 h at 20 ± 2°C. The mixture was then filtered using Whatman's No 1 filter paper and the filtrates were collected for antimicrobial assay. The discs, made up of Whatman's No. 1 filter papers, were soaked in aqueous methanolic extracts of callus and used for antimicrobial assay.

Antimicrobial assay

Pure culture of fish pathogenic microbe *Pseudomonas aeroginosa* (MTCC Accession No. 3451) were procured from IMTECH Chandigarh, India and inoculated into the nutrient agar plate and sub-cultured for 24 h at 36 ± 1°C. The inoculum for anti-microbial assay were prepared aseptically by adding the fresh culture into 1 mL sterile tubes and used for streaking. The antimicrobial assay was performed by disc diffusion assay method using Mueller-Hinton agar (MHA) medium (HiMedia, product code-M173). This medium is best suited for microbial growth studies and gives satisfactory results in every susceptible test as it shows high reproducibility and low in in the content of different types of inhibitors. The medium was prepared by dissolving 38 g MHA medium in 1000 mL distilled water. The medium was autoclaved at 73.23 kg fm^{-2} pressure, 121°C temperature for 15 min. The pH was adjusted to 7.2 ± 0.1. For antimicrobial assay, disc diffusion method was followed on *P. aeroginosa* using the discs prepared from callus extract. Ampicillin and chloramphenicol discs (10 µg $disc^{-1}$) were used as reference. The percent inhibition of callus extract against *P. aeroginosa* with ampicillin and chloramphenicol as reference were calculated as per formula of Singh *et al*. (2023).

$$\text{Percent inhibition (PI)} = \frac{\text{ZOI of test antibiotic} - \text{ZOI of callus extract}}{\text{ZOI of test antibiotic}} \text{ x } 100$$

Where ZOI is zone of inhibition

Chromatography of callus extract

The extract was subjected to the preparative HPLC (Breeze HPLC) with column 2998 Ch1 @ 1.2 nm at flow rate of 200 µL mL^{-1} and retention time 50 min using HPLC grade methanol as solvent along with 0.01% formic acid. The elutes showing maximum antimicrobial efficacy were subjected to LC-MS using HPLC grade methanol as solvent along with 0.01% formic acid. The instrument used was Q-Exactive Plus Biopharma, Thermo Scientific Data Acquisition Software: Thermo Scientific Xcalibur, Version 4.2.28.14 Data Processing Software: Compound Discoverer 3.2 SP1 Column details were SB-C18 RRHD 100 x 2.1 MM, 1.8 microns (Agilent Technologies), METLIN, Agilent database for identification of the compounds.

Statistical analysis

The data were statistically analysed using SPSS v.24.0 following mean of triplicates with standard deviation. All the values indicate mean (N = 3) ± standard deviation considering normal distribution method at P = 0.05.

RESULTS AND DISCUSSION

The explant (entire frond and frond disc) from *Spirodela polyrrhiza,* raised on three basal media (served as control) *viz*., Murashige and Skoog (MS) basal medium, Gamborg B5 basal medium and Schenk & Hildebrand (SH) basal medium, revealed no callus induction. However, when these basal media were supplemented with plant growth regulators (PGRs) like 2,4-D or IBA + BAP depicted callus induction (Table 1). Table 2 depicts the biomass of callus obtained from the fronds and frond discs of *S. polyrrhiza*. Fig. 1 shows the chromatogram of callus extract. The photographs of callus in three basal media fortified with PGRs are given in Fig. 2A-C, whereas Fig. 2D represents antimicrobial efficacy of callus extract against *Pseudomonas aeroginosa*. All the data were statistically analysed with p = 0.05.

Table 1: Callus induction from *Spirodela polyrrhiza* (N = 3) on basal media fortified with PGRs

Explant	Basal media supplemented with PGR	DCI	% CI	Nature of callus
Entire frond	MS + 1.0 µM 2,4-D	21 ± 1.34	25 ± 1.90	Small, brown, hard
	B5 + 1.0 µM IBA + 1.0 µM BAP	NCI	NCI	NCI
	B5 + 1.0 µM 2,4-D	18 ± 1.23	20 ± 1.54	Small, brown, hard
	MS + 1.0 µM IBA + 2.0 µM BAP	20 ± 1.78	20 ± 1.66	Small, brown, hard
	B5 + 1.0 µM IBA + 2.0 µM BAP	18 ± 0.95	40 ± 3.12	Small, brown, friable
	MS + 2.0 µM IBA + 4.0 µM BAP	NCI	NCI	NCI
	B5 + 2.0 µM IBA + 4.0 µM BAP	18 ± 1.12	80 ± 5.78	Medium, brown, hard
Frond disc	MS + 1.0 µM 2,4-D	NCI	NCI	NCI
	B5 + 1.0 µM 2,4-D	21 ± 1.55	20 ± 1.95	Small, brown, hard
	MS + 2.0 µM IBA + 4.0 µM BAP	18 ± 1.12	30 ± 2.55	Medium, brown, hard
	B5 + 2.0 µM IBA + 4.0 µM BAP	18 ± 1.43	60 ± 5.34	Medium, brown, hard
Both type	SH + 2.0 µM IBA + 4.0 µM BAP	20 ± 1.78	60 ± 4.87	Medium to large, brown to black, hard

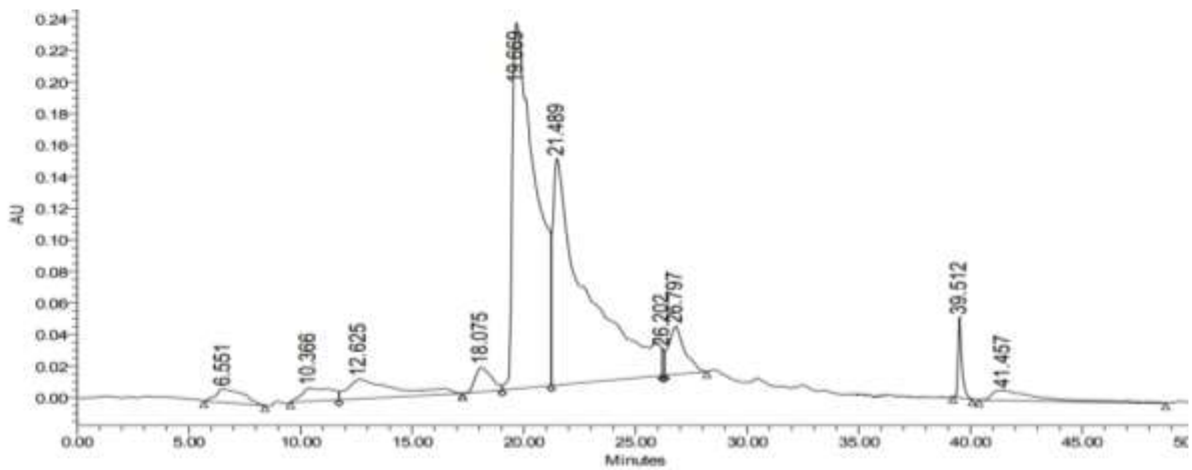
MS: Murashige and Skoog basal medium, B5: Gamborg's B5 basal medium; SH: Schenk & Hildebrand basal medium; IBA: Indole butyric acid, 2,4-D: 2,4-dichlorophenoxy acetic acid, BAP: 6-benzyl aminopurine, DCI: Days to callus induction; %CI: Percent callus induction; NCI - No callus induction

Among the three basal culture media used, MS medium was found least responsive to callus induction in both *S. polyrrhiza* in combination with PGRs used both in terms of days to callus induction (DCI) and percentage of callus (%CI). B5 media was found to induce callus in *S. polyrrhiza* in combination with 2,4-D, whereas BA and IBA were non-responsive to callus induction. SH medium only induced callus growth in *S. polyrrhiza* fortified with two PGRs namely BA and IBA. B5 medium fortified with 2.0 µM IBA+ 4.0 µM BAP showed highest percentage of callus induction in *S. polyrrhiza*. Days to callus induction in B5 media fortified with PGRs were higher along with percentage of callus induction in *S. polyrrhiza*. However, the highest percentage for callus induction for *S. polyrrhiza* were found in SH media fortified with BA and IBA (2:1) long with lower DCI. B5 media fortified BA and IBA in 2:1 ratio showed lower DCI along with higher percentage of callus induction as compared to the 1:1 combination of both IBA and BA for the callus induction of giant duckweed species. Regeneration of callus have been reported previously on MS basal media in combination with PGRs like NAA and 2,4-D supplemented with galactose and sorbitol as carbon source (Li *et al*., 2004). The biomass of callus using entire fronds or frond discs of *S. polyrrhiza* were found to be highest in case of SH media fortified with IBA and BA followed by MS media and B5 media. Least biomass of callus were obtained in MS and B5 media fortified with 2,4-D only, whereas the same basal media fortified with IBA and BA in 2:1 ratio resulted in five-fold increase in callus biomass both from the entire fronds and frond discs as explants. Previous reports showed that modified Huttner Medium (Landolt, 1987) supplemented with ammonium as a sole nitrogen source sustained *in vitro* growth of *S. polyrrhiza* (Vermatt and Hanif, 1998). The relative growth rate (RGR)

Table 2: Comparison of callus biomass from *Spirodela polyrrhiza*, induced on combination of PGR fortified with three types of basal media (N = 3, p = 0.05)

Explants	Medium used	Biomass of callus (mg)
Entire frond	MS + 1.0 µM 2,4-D	6.0 ± 0.05
	B5 + 1.0 µM 2,4-D	9.0 ± 0.06
	MS + 1.0 µM IBA + 2.0 µM BAP	12 .6 ± 0.15
	B5 + 1.0 µM IBA + 2.0 µM BAP	15.5 ± 0.18
	MS + 2.0 µM IBA + 4.0 µM BAP	25.0 ± 0.25
	B5+ 2.0 µM IBA + 4.0 µM BAP	35.0 ± 0.45
	SH + 2.0 µM IBA + 4.0 µM BAP	40.0 ± 0.50
Frond disc	MS + 1.0 µM 2,4-D	8.0 ± 0.12
	B5 + 1.0 µM 2,4-D	10.5 ± 0.25
	MS + 1.0 µM IBA + 2.0 µM BAP	16.0 ± 0.55
	B5 + 1.0 µM IBA + 2.0 µM BAP	18.0 ± 0.25
	MS + 2.0 µM IBA + 4.0 µM BAP	42.0 ± 0.50
	B5 + 2.0 µM IBA + 4.0 µM BAP	45.0 ± 0.45
	SH + 2.0 µM IBA + 4.0 µM BAP	48.0 ± 0.45

MS: Murashige and Skoog Basal media, B5: Gamborg's B5 basal medium; SH: Schenk & Hildebrand basal medium; IBA: Indole butyric acid; 2,4-D: 2,4-dichlorophenoxy acetic acid; BAP: 6-benzyl aminopurine



	RT (min)	Area (µV*sec)	% Area	Height (µV)	% Height
1	6.551	750961	1.92	8532	1.63
2	10.366	763965	1.95	8387	1.60
3	12.625	1859159	4.74	12486	2.38
4	18.075	774848	1.98	15452	2.95
5	19.669	16775471	42.81	231681	44.16
6	21.489	15094980	38.52	143485	27.35
7	26.202	115467	0.29	16900	3.22
8	26.797	1521828	3.88	30316	5.78

Fig. 1: Chromatogram (HPLC) of aqueous methanolic extract from callus of *Spirodela polyrrhiza*

decreased with increase in the concentration of ammonia nitrogen. The maximum amount of biomass or growth rate of *S. polyrrhiza* was observed on the 3rd day of culture in Huttner medium, but the rate decreased with increase in ammonium concentration. pH of media was found to be an important factor for callus induction in duckweeds. When the pH of medium was below 5, all the fronds of *S. polyrrhiza* died (Hilman, 1961). The induction of callus was successful in *S. polyrrhiza* using the root

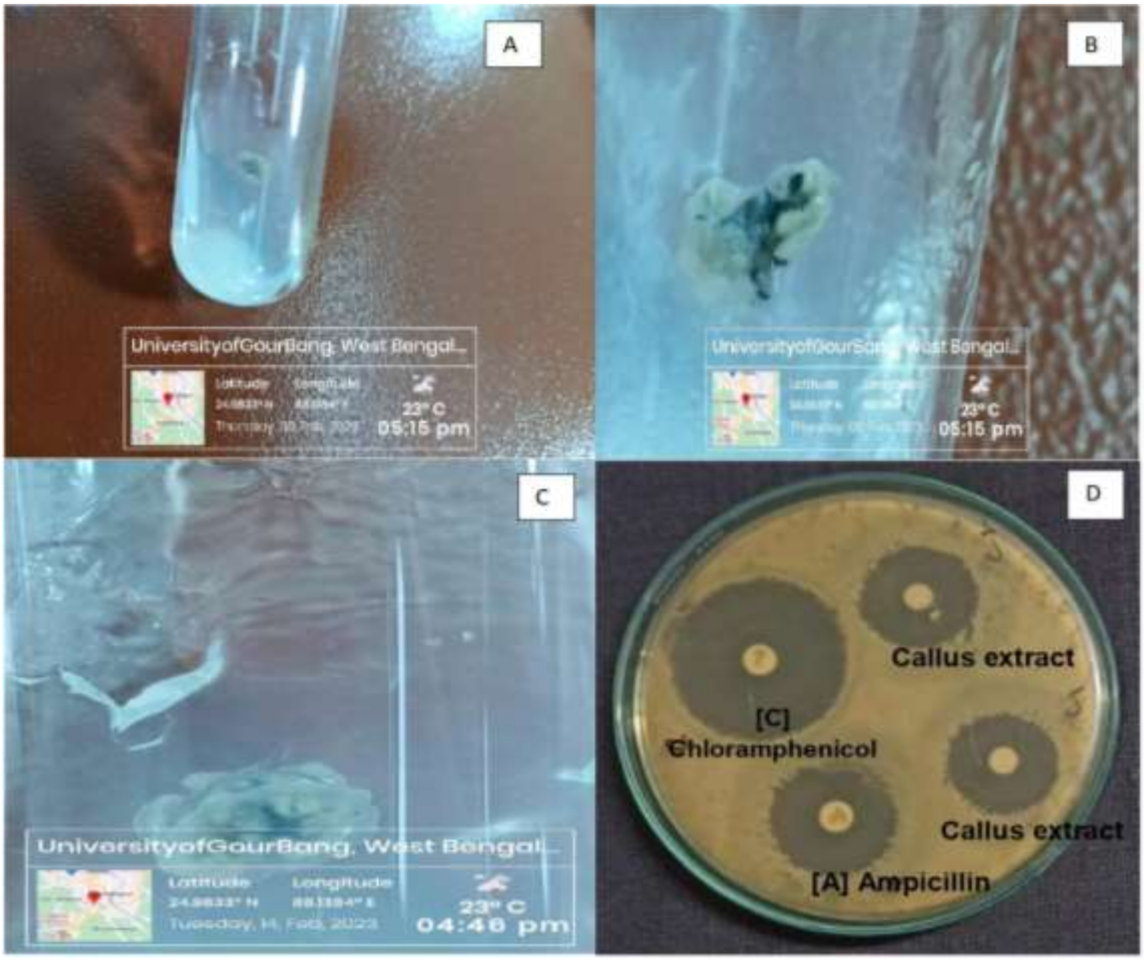


Fig. 2: A) Mature callus of *Spirodela polyrrhiza* (S.) in B5 + 2.0 µM IBA+ 4.0 µM BA, B) Mature callus of *S. polyrrhiza* in SH + 2.0 µM IBA+ 4.0 µM BAP; C) Mature callus of *S. polyrrhiza* in MS + 2.0 µM IBA+ 4.0 µM BAP; and D) Antimicrobial efficacy of callus extract against *Pseudomonas aeruginosa;* C_1 and C_2 represent zone of inhibition induced by callus extract

apical meristem in MS basal media fortified with 2,4 D and thidiazuron. Successful maintenance of callus line was achieved by lowering the level of PGRs for 3 years or more (Huang *et al*., 2018). Callus induction of 92% has been reported in SH and MS basal media using 10 µM NAA and 1 µM 2, 4-D with thidiazurone and sorbitol as carbon source (Wang, 2016).

With the help of disc diffusion method, the anti-microbial property of callus extract was assessed. Ampicillin and chloramphenicol antibiotics were used as control to test the efficacy of callus extract against test microbe *Pseudomonas aeruginosa*. Sterilised paper discs were placed in aqueous methanolic extract for 2 h at room temperature. Two such paper discs were placed on the culture plate using bacterial pure culture of *P. aeruginosa*. along with two antibiotic disc containing ampicillin and chloramphenicol, respectively. After 48 h incubation at 28°C, the zones of inhibition were recorded. Chloramphenicol produced highest zone of inhibition (35.5 mm), followed by ampicillin (24.5 mm). The zone of inhibition of two paper discs were 22.5 and 22.0 mm, respectively (Table 3 and 4). The data were statistically analysed at $p = 0.05$. The study clearly indicated that the callus extracts have considerable potentialities to restrict the growth of *P. aeruginosa* as antimicrobial agent. It is obvious from the percent inhibition of C_1 and C_2 against both chloramphenicol (36.33 and 37.12%) and ampicillin (15.78 and 16.12%) as reference antibiotics. Earlier reports have suggested that ethanolic extracts of *S. polyrrhiza* were rich in flavonoids that prevented the proliferation of adipocytes cells of human beings (Cho *et al*., 2008). The ethanolic extract of *S. polyrrhiza* showed high efficacy against

Table 3: Antimicrobial efficacy of callus extract from *Spirodela polyrrhiza* showing zone of inhibition (mm) against *Pseudomonas aeruginosa* with chloramphenicol (C) and ampicillin (A) as reference. C_1 and C_2 represent the zone of inhibition induced by the callus extract. Values are the mean followed by SD (N = 3, triplicate, p = 0.05)

Antimicrobial agent	Zone of inhibition (mm)
Chloramphenicol (C)	33.7 ± 2.12
Ampicillin (A)	25.5 ± 0.98
Callus extract (C_1)	21.5 ± 0.22
Callus extract C_2)	21.0 ± 0.54

Table 4: Antimicrobial efficacy of callus extracts (C_1 and C_2) against *Pseudomonas aeruginosa* showing percent inhibition with chloramphenicol (C) and ampicillin (A) as reference (N = 3, p = 0.05)

Callus extract	Percent inhibition (%) chloramphenicol as reference	Percent inhibition (%) ampicillin as reference
Callus extract (C_1)	36.43 ± 1.21	15.78 ± 0.67
Callus extract C_2)	37.12 ± 0.98	16.12 ± 0.56

Gram +ve and Gram -ve bacteria. High polarity chromatographic elutes of ethanolic extracts were found to inhibit the *in vitro* growth of three species of *Pseudomonas* sp., two species of *Vibrio* along with *Escherichia coli* with maximum inhibition zones ranging from 13.16 to 14.33 mm (Das *et al*., 2012).

Table 5: Probable compounds responsible for antimicrobial effect of *Spirodela polyrrhiza* Aq met. extract against *Pseudomonas aeroginosa* (MTCC Accession No. 3451)

Name of the compound	Empirical formulae	Molecular weight (Da)	Retention time (min)
Orientin	$C_{21} H_{20} O_{11}$	448.0995	9.28
Genistin	$C_{21} H_{20} O_{10}$	348.3639	10.43
Apigetrin	$C_{21} H_{20} O_{10}$	432.1051	10.42

Chromatogram showed the presence of flavonoids in the extract (Fig. 1). The most effective elute fraction of *S. polyrrhiza* (between retention time 26 to 29 min) were subjected to LC-MS which showed the presence of three phytochemicals in the elute fraction. These phytochemicals were identified as orientin, genistin and apigetrin (Table 5). Genistin is an isoflavone which is mostly found in the members of Leguminaceae and exerts antibacterial, antiviral, anticancerous and antioxidative properties (Mei *et al*., 2019). Using agar diffusion method, the antibacterial activity of genistin was studied against *Bacillus subtilis*, *Enterococcus faecalis*, *E. coli*, *Salmonella typhimurium*, *Shigella sonnei*, *Ps. aeruginosa*, and *Staphylococcus aureus.* Amongst these, the most sensitive were *B. subtilis* producing an inhibition zone of 15-17 mm by agar disk diffusion method (Zlatian *et al*., 2018). Apigetrin is a glycoside which is prevalent in fruits and vegetables and exerts profound antioxidant and anti-inflammatory activity. Hepatic cancer cells were arrested in G2/M stage and undergo apoptosis by modulating cyclin B and CDK when treated with apigetrin (Bhosale *et al*., 2023). Orientin is a flavonoid which is soluble in water and has been isolated from a number of medicinal plants like *Jatropha gossypifolia*, *Ocimum sanctum*, *Passiflora* sp. leaves of *Phyllostachys* sp. etc. Orientin shows mild to moderate antiviral activity against Para virus 3 and Herpes Simplex virus (Lin *et al*., 2004). So, it may be concluded that callus can be induced using sterile fronds of *S. polyrrhiza* in basal media fortified with PGRs. The callus promises to be store house of a number of flavonoids like apigetrin, genistin and orientin which in combination may have exerted considerable antimicrobial effect on *Psuedomonas aeruginosa*. Significant information from greater duckweeds in this regard might be lacking and it seems that the present study may be considered as a pilot research project in future.

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Conflict of interest: The authors have no competing interests relevant to this manuscript.

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