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SUCCESSFUL CALLOGENESIS FROM LEAF AND PETIOLE OF *BERGENIA CILIATA* (Haw.) STERNB AND ANTIBACTERIAL ACTIVITY OF CALLUS EXTRACTS

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Abstract

Medicinal plants have been used for centuries to prevent and treat different health disorders. *Bergenia ciliata* is native to Himalayas and is used traditionally as an antipyretic, an astringent, diuretic and for dissolving kidney and bladder stones. Due to the medicinal value of *B. ciliata,* it has been over-exploited leading to be classified an an endangered species. The current study was designed to see *B. ciliata* response to tissue culturing specifically to callus formation and antibacterial activity of callus extracts. Callus inducing media was supplemented with 2, 4-D and Kinetin. Visible signs of callus formation on cut edges of explants were observed after 13 and 14 days of culturing and leaf showed 95% while petiole depicted 88% callus induction, respectively. Highest dry weight of callus was obtained from petioles (0.61 mg), which was 12% higher than that of dry weight of callus produced by leaf explants on same medium. The ethanolic extract of both petiole and petiole-derived callus demonstrated promising antibacterial activity against six pathogenic bacterial strains. The results suggested that callus from valuable medicinal plants can provide a way to produce essential metabolites and help in the conservation of the natural population of endangered plants. In future, evaluating biochemical composition of callus and plants extracts will enable us to find new dimensions to get maximum benefits from *in-vitro* technique.

Key words: Antibacterial; Callus induction; Callus inducing media; Metabolites; Plant growth regulators.

Introduction

Health has always been a priority in the known history of modern world. The utilization of plants and their resources for combating various ailments predates written history and they are still in use all over the world even in the prime era of synthetic medicines (Sulaiman *et al.*, 2015). People living in under-developed countries still rely on plants as primary source of treatment. Integrated therapy is attaining popularity in which patients are treated with different medicines (Kon & Rai, 2012). Plants have great diversity to synthesize variety of metabolites/compounds of medical importance with great ease as compared to laboratory synthesis. Demand of plant based medicine will remain high because various molecules cannot be prepared synthetically. Pakistan is also very rich in the botanical wealth and has a variety of aromatic and medicinal plants because of its exclusive geographical distribution due to multiple ecological regions, varied climatic and soil conditions (Ghulam *et al.*, 2016).

Bergenia ciliata belongs to the family Saxifragaceae and locally known as *Pakhanbhedin* (Nepal), *Butpewa* (Pakistan) and *Paashaanbhed* (India) (Neha *et al.*, 2013). It is a perennial plant having hairy leaves, found in Himalayas and is therapeutically effective against kidney stones, ulcers, spleen enlargement and dysantry (Bayhatti *et al.*, 2010). The important phyto-constituents present in *B. ciliata* include flavonoides, tannins, sterol, bergenin, gallic acid, mucilage, and β-sitosterol (Shrestha & Bijaya; 2011). Traditionally, Bergenia rhizome is used to treat fractured bones, fresh cuts, wounds, diarrhoea, pulmonary infections, vomiting, fever and cough. It is also used for the treatment of heart disease, haemorrhoids, stomach disorders and ophthalmia (Mithilesh *et al.*, 2016). The alcoholic extract and the rhizomes of *B. ciliata* revealed important anti-inflammatory, analgesic, anti-urolithiatic, anti-bacterial as well as diuretic attributes (Sinha *et al.*, 2001, Neha *et al.*, 2013). Continuous demand of medicinal plants to meet the requirement of local and international market poses various threats to the existence of different plant species. The non-professional uprooting of valuable medicinal plants including *B. ciliate* has led to nearly extinction of this species. Unfortunately, there is no consideration among the users of such important plants to rehabilitate the medicinally important plants population in nature Alternative propagation methods for rapid multiplication and conservation of the medicinal plants should be adopted.

Plant Tissue culture technique has been used as an alternative for the vegetative propagation of numerous important plants (Kumar & Thomas, 2012) and effectively employed for the conservation of endangered plant species as well as production of pathogen free plants (Sarwat *et al.*, 2018). *In vitro* propagation and callus induction responses for the conservation purpose have been studied in various medicinal plants such as *Labisia pumila* (Anna *et al.*, 2013), *Elephantopus scaber* Linn., (Jyoti & Dennis, 2015) and *Aquilegia nivalis* (Mudasar *et al.*, 2016). Moreover, important metabolites can be produced at commercial scale using cell and tissue culture. Therefore, it becomes imperative to initiate dedicated study to commence callus production from important medicinal plants. Additionally, it is also desired

to evaluate the callus extracts to have similar metabolic profiles as compared to naturally grown plants. The callus produced from any tissue of plant may have important secondary metabolites, which will pave the way to obtain important metabolite without compromising natural population of plants.

Infectious diseases are the leading cause of death in the world and antibiotic resistance has also become a global concern. Emergence of multidrug resistant pathogens reduces the clinical efficacy of many existing antibiotics. The most notorious bacteria include, but are not limited to, extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-EC), *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) (Bishnu *et al.,* 2015). Thetherapeutic options for these pathogens are extremely limited and physicians are forced to use expensive drugs (Bishnu *et al.,* 2015). Therefore, the situation prompt a revival in research of the antimicrobial role of plants against resistant strains due to comparable safety and efficacy.

In this study, we aimed to evaluate tissue culture responses of *B. ciliata* plant. Callus induction was successfully achieved. The extracts of callus depicted promising antimicrobial activities against pathogenic bacteria.

Materials and Methods

Plant material and explants preparation: The plant material for research purpose was collected from the pool of medicinal plants maintained at COMSATS, University Islamabad, Abbottabad Campus, KP, Pakistan. The third and fourth leaves from top were taken for callus because they were fully expanded and suitable for callus induction. The explants were prepared from the nascent leaves and corresponding petioles. Explants from leave and petioles of 6-8 mm size were prepared for callus induction experiments. The leaves and petioles were thoroughly washed under running tap water followed by rinsing with autoclaved distilled water. The washed explants were surface sterilized by treating with 50% commercial bleach. Subsequently, they were washed with 0.1% $HgCl_2$ for 5 minutes. The remnants of bleach and $HgCl_2$ were removed by washing the explants with autoclaved distilled water three times for 5 minutes each.

Media preparation for callus and bacterial culture: For callus induction studies, full strength Murashige and Skoog (1962) media was prepared and pH (5.8) was maintained. The MS media was supplemented with 3% sucrose and 0.6% plant agar. Different plant growth regulators (PGR) with varying concentrations were added into the autoclaved media (Table 1). LB media was prepared for bacterial culturing and pH (7.0) was maintained.

Culturing of explants on different callus inducing media: The sterilized explants were cultured on different callus inducing media (CIM). The petri plates were sealed and kept in dark at 25°C. The petri-plates were observed after every two days to monitor any contamination. The contaminated explants were removed from the healthy explants. The healthy explants were considered as qualified explants for callus induction. All subsequent observations and data record were taken from these explants. The media was changed after every two weeks. Data related to percent callus induction, days to callus induction and weight of callus was recorded. For weight of callus, 10 explants from each replicate per treatment were pooled and weighed after drying.

Table 1. Different concentrations of plant growth regulators used in MS media.

Media	Ex-plant	Plant growth regulators used in MS media
CIM_1	Leaf, Petiole	2,4-D (2mg/L)
CIM_2	Leaf, Petiole	2,4-D (3mg/L)
CIM_3	Leaf, Petiole	2,4-D (4mg/L)
CIM_4	Leaf, Petiole	2,4-D (2mg/L + Kn 2mg/L)

Table 2. Pathogenic bacteria used to evaluate antibacterial activities of callus extract.

S. No.	Name of the pathogenic bacteria
1.	*Pseudomonas aeruginosa*
2.	*Staphylococcus aureus*
3.	*Acinetobacter baumannii*
4.	*Methicillin-resistant Staphylococcus aureus*
5.	*Streptococcus agalactiae*
6.	*Escherichia coli*

Extraction of metabolites from callus and fresh plant samples: The petiole part of *B. ciliata* was obtained from intact plant of same aged leaf as used for callus induction experiments. The fresh petiole as well as the callus (derived from petioles) were dried in drying oven at 48°C until complete removal of water contents. The temperature was set 48°C to avoid metabolite deterioration. Equal amount of powdered petiole and callus derived biomass was extracted in absolute ethanol and kept overnight. The fluid was sonicated to disrupt the cells and the cell debris was removed from the extraction fluid. Afterwards the extract was set to air dry and ethanol was evaporated. The dried extracts were dissolved in ethyl acetate and used for thin layer chromatography (TLC) and antibacterial activity.

Analysis of antimicrobial activity: The six different pathogenic bacterial strains were obtained from Army Medical College, Rawalpindi, Pakistan (Table 2). The bacterial strains were cultured in nutrient broth for utilization in assay. Agar-well diffusion method was used to evaluate antimicrobial activity of extracts as described by Okeke *et al.,* (2001). Briefly, wells were made in agar nutrient plates using sterilized borer and bacterial culture was spread over the plates. Extracts (50 µL) dissolved in ethyl acetate, was added into the wells while ethyl acetate was used as control. The treated plates were kept in incubator at 37°C. The zone of inhibition around the wells (where bacterial growth didn't appear) was determined after 24 h. The experiment was replicated thrice.

Statistical analysis

All the experiments were replicated three times at least. The means and the *t- test* was employed by using Microsoft excel 2010. The *p*-value to evaluate the significance was set as 0.05.

Results

Callus induction responses of *B. ciliata* to varying plant growth regulators: Differential responses of callus induction were observed in leaf and petiole explant of *B. ciliata* against 4 different callus inducing media (Table 1). Highest callus induction (%) was achieved in leaf (95%) and petiole (88%) explants when cultured on CIM_4 (Fig. 1). The response of callus induction was lowest against CIM_1 medium in which leaf and petiole depicted 35 % and 42% callus induction, respectively (Fig. 1). Petiole-explants showed significantly highest percent callus induction when cultured on CIM_1 and CIM_2 as compared to leaf-explants (Fig. 1). Visible signs of callus formation on cut edges of explants were observed and leaf and petiole explants showed callus signs after 13 and 14 days of culturing, respectively (Fig. 2). Contrary to this, highest days (19 and 18) were taken by leaf explants on CIM_3 and CIM_2, respectively. Non-significant differences were found in days to callus induction responses among all tested explants and media used.

Analysis of biomass during *In vitro* culturing: Petiole explants proved to be best in cell proliferation and biomass accumulation during *In vitro* culturing. Highest dry weight of callus was yielded by petioles (0.61 mg), cultured on CIM_4 medium, which was 12% higher than that of dry weight of callus produced by leaf explants on same medium (Fig. 3). On CIM_1, CIM_2 and CIM_3, petiole explants produced 0.42, 0.49 and 0.53 mg of callus which was 21% higher than leaf explants (Fig. 3). A representative photograph also depicts the difference in biomass accumulation in leaf and petiole explants (Fig. 4).

Extraction of metabolites and antimicrobial activity assay: Consortium of metabolites was extracted from petiole and petiole-derived callus as described in preceding section. The extracts were dissolved in ethyl acetate and subjected to TLC. The TLC plate depicted similar band patterns in petiole and callus extract upon exposing to UV light (data not shown). The extracts were used to evaluate antimicrobial activity against 6 pathogenic bacterial species (Fig. 5). Ethyl acetate was used as control, which didn't show any activity against any bacterial species, however, both callus and petiole extracts depicted antibacterial activity albeit at different levels (Fig. 5). The callus extract demonstrated higher antibacterial activity than petiole extracts against *P. auriginosa, S. aureus, S. agalactai* and *E. coli*, in which activity against *P. aeruginosa* and *S. agalactei* was statistically significantly higher. Contrary, petiole extracts showed higher activity against *A. buawami* and *methicilin resistant S. aureus* (*MR. Staph*).

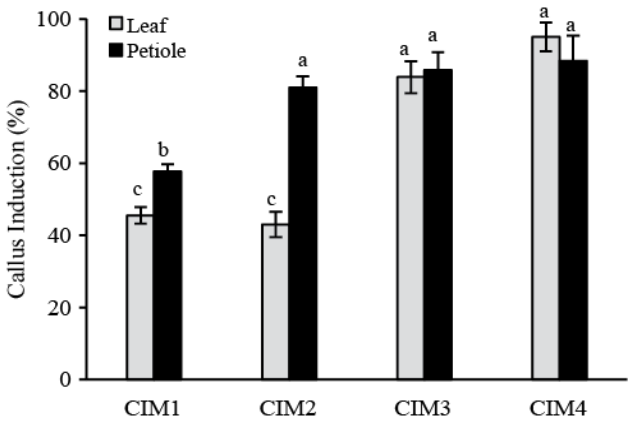

Fig. 1. Percentage of callus induction in different explants of *Berginia ciliata*. Leaf and petiole explants were cultured on media supplemented with different plant growth regulators named as CIM1-4. Percentages were calculated on the basis of explants which showed visible signs of callus formation to those where callus was not visible. Data is expressed as the mean ± SD of three replicates. Bars labelled with same letter are not significantly different ($p<0.05$) according to Student's t-test.

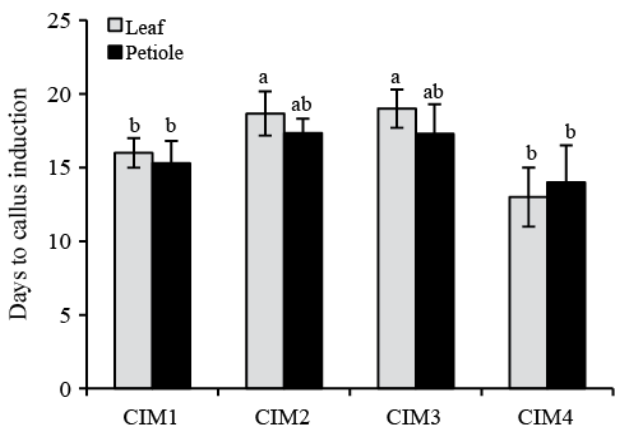

Fig. 2. Days to callus induction after first culture on different callus inducing media, CIM1-4. Days were calculated after visual observation of clear sign of callogenesis on cut surfaces of leaf and petiole explants. Data is expressed as the mean ± SD of three replicates. Bars labelled with same letter are not significantly different ($p<0.05$) according to Student's t-test.

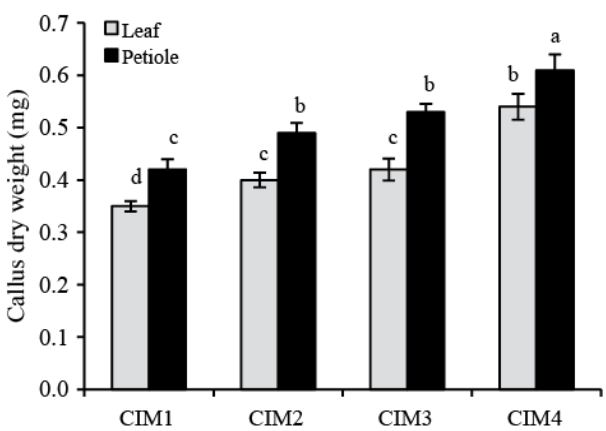

Fig. 3. Dry weight of callus produced after culturing on different callus inducing media, CIM1-4. Callus of each replicate was dried at low temperature and weighed. Data is expressed as the mean ± SD of three replicates. Bars labelled with same letter are not significantly different ($p<0.05$) according to Student's t-test.

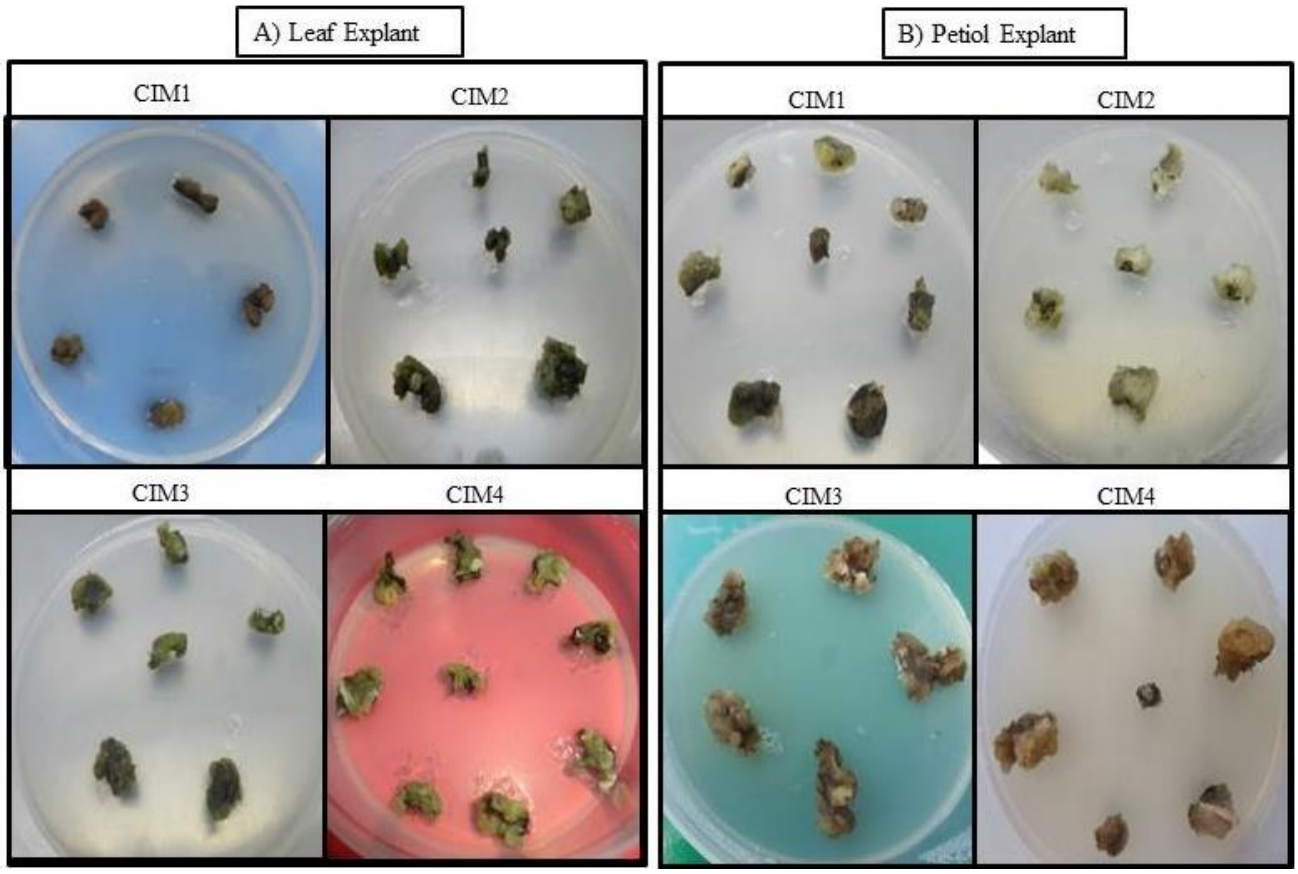

Fig. 4. Representative photographs depicting callus formation on explants. A) Callus induction on leaf explants after culturing on different callus inducing media (CIM1-4), B) Callus induction on petiole explants after culturing on different callus inducing media (CIM1-4).

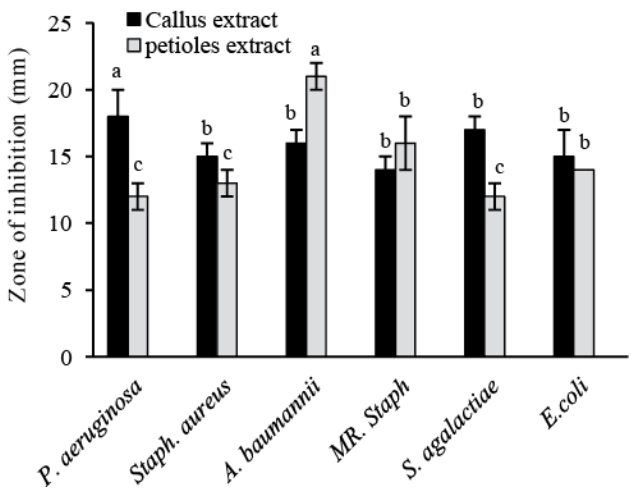

Fig. 5. Antibacterial activity of plant and callus derived extracts against 6 pathogenic bacterial strains. Callus extracts and petiole extracts were used in agar well diffusion assay and compared with control (ethyl acetate). Data is expressed as the mean ± SD of three replicates. Bars labelled with same letter are not significantly different ($p<0.05$) acording to Student's t-test.

Discussion

Over exploitation of medicinal plants has posed serious threats to their existence. *In-vitro* techniques can be employed to rescue the endangered species or callus production can be used for extraction of important metabolites (Rand *et al.*, 2010). Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and other industrial materials (Jian *et al.*, 2005). Organized cultures including treatment with various elicitors and signal compounds effectively promote the production of a wide range of plant secondary metabolites, both *in-vivo* and *in-vitro* (Zhang *et al.*, 2004, Srivastava *et al.*, 2007, Smetanska; 2008). Many valuable chemicals and metabolites are produced through plant cell and tissue culture in the same way as in parent plants (Baskaran *et al.*, 2014). These metabolites and othe bichemicals including alkaloids, tannins and flavonoids have medicinal importance and act aginst various microorgansims (Manasa *et al*., 2016).

Bergina ciliata has various medicinal uses and possesses an important compound known as bergenin, which is reported to have hepato-protective and immune-modulatory activities (Dharmender *et al.*, 2010). In current study, leaf and petiole explants responded differentially to same experimental conditions i.e. MS media supplemented with 2, 4-D and kinetin. However in both cases, sequential increase in 2, 4-D concentration increased the callus induction parameters in similar fashion. The results were in accordance with the Gupta *et al*., (2010) that 2, 4-D can be employed successfully for callus induction experiments. Auxin is involved in many plant regulatory processes such as plant growth and development (Dharmasiri *et al.*, 2005). Auxin is more persistent and resist to heat damage, doesn't degrade quickly in stock solutions and relatively inexpensive. Like auxin, cytokinin is also a key regulator for various aspects

of plant growth and development. Auxins and cytokinins are known to interact at different levels in synergistic, antagonistic or additive ways to produce or regulate physiological effects (Amoo *et al.*, 2013) such as the formation and maintenance of meristem (Ying *et al.*, 2011). Auxins are absorbed by the explants within hours which explants may utilize to promote cell division later on and cytokinins are used in tissue culture to expedite and maintain cell division (Anum *et al.*, 2016) Therefore, the combination of auxin with cytokinin (kinetin) also showed improved callus induction. 2,4-D and kinetin has been proved to be best growth regulators for callus induction (Ramin *et al.*, 2014).

Plant extracts may act synergistically with conventional antibiotics for provision of enhanced antibacterial activities (Milenković *et al.*, 2015). However, overall antibacterial activities of callus extracts were better than plants extracts. The callus extracts depicted higher microbial growth inhibition to 4 out of 6 tested bacterial species. Zabta *et al*., (2019) also tested the antibacterial activity in *Calligonum polygonoides* L. and *Typha latifolia* L. and reported promising antibacterial activity against *P. auriginosa* and *E. coli*. Fethi *et al*., (2016) documented the restricted growth of *S. aureus* and effective therapeutic results in callus. Various metabolites are also produced in varying concentration in the callus as in intact plant (Singh & Sudarshana, 2003) and antimicrobial properties are afected by secon dary metabolites (Fethi *et al.*, 2016). The detail biochemical investigation can unreveal the underlying metabolite potential in *B. ciliate.*

Conclusion

Our investigations instigate that callus from important medicinal plants can be considered as source to produce commercially important metabolites and save the endangered plant population. However, in depth plant metabolite profiling is necessary to achieve a solid conclusion. We are in process to evaluate biochemical composition of callus and plants extracts, which will give new dimensions to harvest maximum benefits from *In vitro* technique in future.

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