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In vivo and *in vitro* phytochemical and antibacterial efficacy of *Baliospermum montanum*(Willd.) Muell. Arg.

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ABSTRACT

Objective: To evaluate the phytochemical and anti-bacterial potential of mother plants *in vivo* and *in vitro* derived callus of *Baliospermum montanum (B. montanum)* (Willd.) Muell.-Arg. leaves and root. **Methods:** The *in vitro* derived rootlets and leaves segments of *B. montanum* were cut into 0.5-0.7 cm in length and cultured on Murashige and Skoog solid medium supplemented with 3% sucrose, gelled with 0.7% agar and different concentration of 2, 4-D either alone or in combinations. The preliminary phytochemical screening was performed by Harborne method. Antibacterial efficacy was performed by well diffusion method and incubated for 24 h at 37 ℃. **Results:** The highest percentage of callus formation (leaves segments 86.9±0.56; root segments 78.7±0.51) was obtained on Murashige and Skoog's basal medium supplemented with 3% sucrose and 2.0 mg/L of 2, 4-Dichlorophenoxy acetic acid. The phytochemical study revealed the high quantity presence of steroids, triterpenoids, glycosides, saponins, alkaloids, flavanoids, phenolic compounds, tannins, sugars etc of root and leaves derived calli. The ethanol extract of leaves segment derived calli of *B. montanum* showed the maximum solubility and antimicrobial activity with the MIC ranged from 100 to 200 μL. **Conclusions:** The preliminary phytochemical study confirmed that the calli mediated tissues showed the higher percentage of metabolite constituents and extraction value compared to the *in vivo* leaves and roots. The present study observation suggested that a possibility to establish high yielding genotypes by *in vitro* culture for production of medicinally important bioactive compounds.

1. Introduction

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides[1]. Studies on plant secondary metabolites have been increasing over the last 50 years. A large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. Many pharmaceutical compounds are isolated from the secondary metabolites of plants for example; digitalis, L-dopa, morphine, codeine, reserpine, and the anticancer drugs vincristine, vinblastine and taxol used in treatment of ovarian and breast cancers[2]. Moreover, different national and international pharmaceutical companies are utilizing such plant based formulations in treatment of various diseases and disorders worldwide[3-5]. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases[6,7]. Recently, much attention has been directed toward extracts and biologically active compounds isolated from popular plant species[8,9]. The global demand of plant origin bioactive compounds is very high, but not possible to fulfill by field grown plants. An attractive and very promising alternative system for commercial exploitation is plant cell cultures thereby producing high yield compared to field grown plants[10,11]. Plants may be considered as a famous chemical factory for biosynthesis of a huge array of secondary metabolites[2]. *In vitro* plant cultures often produce secondary metabolites in quantities equal to those produced by plants growing in nature. To date, only a few plant metabolites have been produced via cell culture

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production in industrial scale. In a few cases, cell cultures have been found to produce higher levels of secondary metabolites than the differentiated mother plant itself[12,13].

Baliospermum montanum (*B. montanum*) (Willd.) Muell.-Arg. is a vulnerable medicinal plant belonging to the family Euphorobiaceae. Root, leaf and seeds of the plants are used medicinally. The root contains phorbol ester belonging to dipterene hydrocarbon viz., montanin, baliospermin, 12-deoxyphorbol 13-palmitate, 12-deoxy-5 β -hydroxyphorbol 13-myristate and 12-deoxy-16-hydroxyphorbol 13-palmitate. Leaves contain 8-sitosterol, 8-D-glucoside and hexacosamol. The presence of steroid, terpenoids and flavanoids is also reported from the plant[14]. The root is acrid, thermogenic, purgative, anti-helmintic, carminative and anti-inflammatory. They are useful in abdominal pain, constipation, calculus, piles, helminthic manifestations, scabies, skin disorders, wound and jaundice[15]. Root paste is applied to painful swellings and piles. Leaves cure asthma and bronchitis. They are burgative and also used for dropsy. Seeds are drastic burgative, rubifacient, hydragogue and stimulant that are useful in inflammations and flatulence. Seeds are also used in snakebite[16]. The plant is used for the treatment of abdominal tumours and cancer[17,18]. The alcohol extract of *B. montanum* stimulates cell-mediated immune system by increasing neutrophil function[19]. The multiple use of this important herb has led to its indiscriminate collection. Almost the entire commercial requirement is met solely from the wild natural populations resulting in its listing as a threatened plant. Due to its high demand resulted over exploitation from the wild and leads to depletion of this important medicinal plant and this necessitates create an alternative method for propagation to fulfill the requirements. Micropropagation of *B. montanum* was reported by Johnson and Manickam[20], Geroge *et al*[21] and Sasikumar *et al*[22], *Baliospermum axillare (B. axillare)* by Singh *et al*[23]. To keep pace with the growing demand of this herb, evaluation and utilization of cell culture system as an effective alternative source seems logical as it has already proved successful in many other cases[10]. The main purpose of this study is to evaluate the phytochemical and anti-bacterial potential of mother plants *in vivo* and *in vitro* derived callus from *B. montanum* leaves and rootlets.

2. Materials and methods

2.1. Callus induction

The *in vitro* derived rootlets and leaves segments of *B. montanum* were cut into 0.5-0.7 cm in length and cultured on Murashige and shoog[24] solid medium supplemented with 3% sucrose, gelled with 0.7% agar and different concentration of 2, 4-D either alone or in combinations. The pH of medium was adjusted to 5.8 before autoclaving at a pressure of 1.06 kg/cm^2 (121 ℃ for 15 min). For callus induction and proliferation, the cultures were incubated with 16/8 h photoperiod under white fluorescent tubes (1 500 lux) at 25 ℃±2 ℃ with 80% relative humidity. Each and every experiment was performed with ten replicates and repeated thrice. The callus cultures were maintained for a period of over 10 months by periodic sub-culturing with 2 weeks intervals on to fresh multiplication medium.

2.2. Phytochemical analysis

In vitro derived callus (leaves and rootlets) of *B. montanum* were dried in the hot air oven and powdered using the electric homogenizer. The powdered samples were extracted with 150 mL of solvent (hexane, chloroform, ethanol, isopropanol and petroleum ether) for 8-12 h by using the soxhlet apparatus[25]. The preliminary phytochemical screening was performed by Harborne method[26].

2.3. Antibacterial activity

The crude extracts of *B. montanum* leaves and rootlets *in vivo* and *in vitro* tissue (calli) derived extracts were concentrated and subjected for their antibacterial activity against the selected pathogenic bacteria. Stock cultures were made fresh every seven days on agar slants during this scheme of work. Pure bacterial cultures, namely *Staphylococcus aureus (S. aureus)*(ATCC 6538), *Pseudomonas aeruginosa (P. aeruginosa)*(isolates from diseased fish), *Klebsiella aerogenes (K. aerogenes)*(isolates from diseased fish), *Aeromonas formicans (A. formicans)*(isolates from diseased fish), *Vibrio chloreae* (*V. chloreae*), *Bacillus subtilis (B. subtilis)* (ATCC 10707) and *Escherichia coli (E. coli)*(ATCC 35218) were maintained on nutrient broth at 37 ℃ for 24 h. Different concentrations of extracts ranging from 25 to 200 μL were used for bacterial sensitivity test. Antibacterial efficacy was performed by well diffusion method and incubated for 24 h at 37 ℃[27]. The inhibition zone and antibacterial activity against the pathogenic bacteria were recorded. The experiments were repeated in triplicate and the results were documented. Streptomycin was used as a positive control.

3. Results

The *in vitro* derived young leaves and root segments of *B. montanum* were cultured on MS medium segmented with 2, 4-D alone or in combination with kin showed the callus induction with varied degree (Table 1). Highest percentage of callus proliferation (78.7±0.51 on root and 86.9±0.56 on leaves) was observed in Murashige and Skoog's basal medium supplemented with 3% sucrose and 2.0 mg/L of 2, 4-D (Table 1). Three types of callus viz., friable, semi-friable and compact were obtained. The explants cultured on MS medium augmented high concentration of auxins showed the compact and dark yellowish brown callus with low proliferation percentage. The white semi friable and friable callus was showed highest rate of multiplication (Table 1).

The preliminary phytochemical study confirmed that the calli mediated tissues showed the higher percentage of metabolite constituents and extraction value when compared to the *in vivo* leaves and rootlets. The phytochemical study documented the high quantity of steroids, triterpenoids, glycosides, saponins, alkaloids, flavanoids, phenolic compounds, tannins, sugar, *etc* in leaves and rootlets derived calli. Different kinds of solvents were used for extraction, of which ethanol extracted solvents showed maximum values (9/13) compared with other solvents extracts (Table 2).

Table 1

Effect of 2, 4-D on callus production from the leaves and root segments of *B. montanum*.

MS medium + plant growth regulator (2, 4-D) (mg/L)	Percentage of callus induction (%)		Type of callus	
	Roots	Leaves	Leaves	Roots
0.0	00.00 ± 0.00	00.00 ± 0.00	NIL	NIL
0.5	38.60 ± 0.74	45.70 ± 0.83	Friable	Semi-friable
1.0	49.70 ± 0.97	57.80 ± 0. 87	Friable	Semi-friable
1.5	63.40 ± 0.86	71.40 ± 0.74	Friable	Semi-friable
2.0	78.70 ± 0.51	86.90 ± 0.56	Friable	Semi-friable
2.5	63.70 ± 0.78	64.80 ± 0.54	Semi-friable	Semi-friable
3.0	53.40 ± 0.81	48.30 ± 0.48	Semi-friable	Semi-friable

Table 2

Phytochemical screening of different solvents extract of *B. montanum*.

Tests	Leaves					Roots					Leaves callus					Roots callus				
	Et	H	Ch	Ip	PE	Et	H	Ch	Ip	PE	Et	H	Ch	Ip	PE	Et	H	Ch	Ip	PE
Flavonoids	++	+	++	−	−	+	−	+	+	−	+++	+	+++++	−	−	+	−	+++	+	−
Glycosides	++	+	+	−	+	++	−	++	+	−	+++	++	++	−	+++	+++	−	++++	++	−
Sugars	+	+	++	−	+	+	−	++	++	−	+	+	+++	−	++	+	−	+++	++	−
Alkaloids	+	−	++	+	+	++	−	+	+	+	++	−	+++	++	++	+++	−	+++	+	++
Phenols	++	−	+++	+	+	+	−	+	+	+	+++	−	+++++	+	+	++	−	++	++	+
Tannins	−	−	++	+	+	++	+	++	−	+	−	−	++	+	++	+++	+	++++	−	++
Saponins	+	+	++	−	−	+	−	++	−	+	++	++	++	−	−	+	−	++	−	+
Triterpenoids	++	+	++	−	−	+	+	+++	+	−	++	++	++	−	−	+++	+	+++	+	−
Steroids	+	−	++	+	−	+	−	++	−	+	+	−	+++	+	−	+	−	+++	−	++

+ indicates the degree of presence, −presents absence. Et −ethanol, Ch − chloroform, H −Hexane, Ip −isopropanol and PE −petroleum ether.

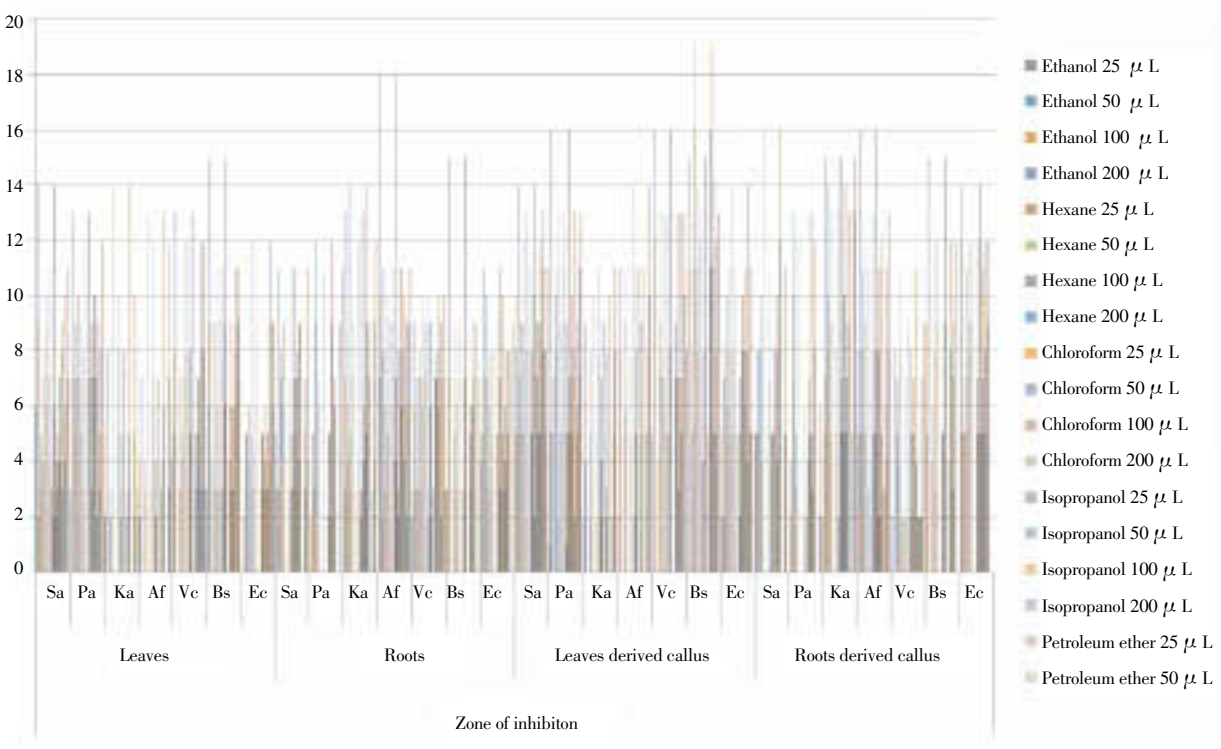

Figure 1. *In vivo* and *in vitro* antibacterial efficacy of *B. montanum* (Willd.) Muell.-Arg.
Sa-*S. aureus*, Pa-*P. aeruginosa*, Ka-*K. aerogenes*, Af-*A. formicans*, Vc-*V. chloreae*, Bs-*B. subtilis*, Ec-*E. coli*.

4. Discussion

The result of the present study revealed that the antibacterial efficacies of ethanol, chloroform, hexane, isopropanol and petroleum ether extracts of leaves and leaves segment derived calli and root and rootlets derived calli extracts of *B. motanum* was diverse in effectiveness which may be attributed to the presence of the secondary metabolites. The ethanol extracted solvents showed the maximum bio-efficacy compared with other solvents due to

the presence of more compounds such as saponins, steroids, tannins, phenolics, triterpenoids, alkaloids and flavanoids. Results of the present study are directly correlated with the previous observations[2,4,12,13,28-31]. Both hexane and petroleum ether extracts were found to be ineffective on the selected pathogenic bacteria, due to the presence of less active compounds saponins, steroids and alkaloids. Similar kind of observation was studied in various plants viz., *Rauvolfia tetraphylla* and *Holostemma ada-kodien* and *Passiflora edulis* leaf and callus extracts[12,32]. The various extracts of *in vivo* leaves and leaves segments derived calli showed the inhibition against the selected pathogenic bacteria. The earlier observations on *Hypericum perforatum* and *Mimosa hamata* leaf and callus extract demonstrated significant antibacterial and antimicrobial activity[14,25]. The present observation augments the previous phytochemical and bio-efficacy studies on cell cultures. The methods developed in this work make possible for the low volume and high potential production of active principles under *in vitro* condition in short duration with less amount of explants utilization. The present study result demonstrated that *in vitro* leaves derived calli increased significant levels of secondary metabolites productivity compared to the field grown plants. The present study observation suggested that a possibility to establish high yielding genotypes by *in vitro* culture for production of medicinally important bioactive compounds.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Balandrin MJ, Klocke JA. Medicinal, aromatic and industrial materials from plants. In: Bajaj YPS. *Biotechnology in Agriculture and Forestry. Medicinal and Aromatic Plant*. Heidelberg: Springer-Verlag; 1988, p. 1-36.

[2] Rao RS, Ravishankar GA. Plant cell cultures: chemical factories of secondary metabolites. *Biotechnol Adv* 2002; **20**: 101-53.

[3] María del Rayo Camacho-Corona, Juan Manuel de Jesús Favela-Hernández, Omar González-Santiago, Elvira Garza-González, Gloria María Molina-Salinas, Salvador Said-Fernández, *et al*. Evaluation of some plant-derived secondary metabolites against sensitive and multidrug-resistant mycobacterium tuberculosis. *J Mex Chem Soc* 2009; **53**(2): 71-5.

[4] Arafeh RM, Shibli RA, Mohsen Al-Mahmoud, Shatnawi MA. Callusing, cell suspension culture and secondary metabolites production in persian oregano (*Origanum vulgare* L.) and arabian oregano (*O. syriacum* L.). *Jordan J Agric Sci* 2006; **2**(3): 274-81.

[5] Vineesh VR, Fijesh PV, Jelly Louis C, Jaimsha VK, Jose Padikkala. *In vitro* production of camptothecin (an anticancer drug) through albino plants of *Ophiorrhiza rugosa* var. decumbens. *Curr Sci* 2007; **92**(9): 1216-8.

[6] Doughari JH. Antimicrobial activity of *Tamarindus indica* Linn. *Trop J Pharm Res* 2006; **5**(2): 597-603.

[7] Maghrani MN, Zeggwah JM, Eddouks M. Antihypertensive effect of *Lepidium sativum* in spontaeneously hypertensive rats. *J Ethnopharam* 2005; **102**(1&2): 193-7.

[8] Ben Sassi AF, Barzallah-Skhiri, Aouni M. Investigation of some medicinal plants from Tunisia for antimicrobial activities. *Pharma Biol* 2007; **15**(5): 421-8.

[9] Coruh I, Gornez AA, Ercisli S. Total phenolics, mineral elements, antioxidant and antibacterial activities of some edible wild plants in Turkey. *Asian J Chem* 2007; **19**(7): 5755-62.

[10] Bourgaud F, Nguyen C, Guckert A. XXII Psoralea sps: *In vitro* culture and production of furanocoumarins and secondary metabolites. Berlin Heidelberg: Springer-Verlag; 1995, p. 388-411.

[11] Shilpa K, Varun K, Lakshmi BS. An alternate method of natural drug production: eliciting secondary metabolite production using plant cell culture. *J Plant Sci* 2010; **5**(3): 222-47.

[12] Karmarkar SH, Keshavachandran R, Augustin A. Biochemical evaluation of root tubers and *in vitro* induced callus of adapathiyan (*Holostemma ada-kodien* k.schum.). *J Trop Agric* 2001; **39**: 108-10.

[13] Shinde AN, Malpathak N, Fulzele DP. Induced high frequency shoot regeneration and enhanced isoflavones production in *Psoralea corylifolia*. *Rec Nat Prod* 2009; **3**(1): 38-45.

[14] Pasqua G, Pinarosa A, Monacelli B, Santamaria AR, Argentieri MP. Metabolites in cell suspension cultures, calli and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. *Plant Sci* 2003; **165**: 977-82.

[15] Shama PC, Yelne MB, Dennis TJ. *Database on medicinal plants used in Ayurveda*. New Delhi: Central Council for Research in Ayurveda and Siddha; 2000.

[16] Warrier PK, Nambiar VPK, Ramankutty C. *Baliospermum montanum (Willd.) Muell-Arg, in Indian medicinal plants*. Madras: Orient Longmen Limited; 1996.

[17] Sivarajan VV, Balachandran I. *Ayurvedic drugs and their plant sources*. New Delhi: Oxford and IBH Publishing Co. Pvt. Ltd; 1994.

[18] Anonymous. *The wealth of India, raw materials*. New Delhi: CSIR; 1988.

[19] Wadekar RR, Agrawal SV, Tewari KM, Shinde RD, Mate S, Patil K. Effect of *Baliospermum montanum* root extract on phagocytosis by human neutrophils. *Int J Green Pharm* 2008; **2**: 111-3.

[20] Johnson M, Manickam VS. *In vitro* micropropagation of *Baliospermum montanum* (Willd.) Muell.-Arg.- A medicinal plant. *Indian J Exp Biol* 2003; **41**: 1349-51.

[21] George S, Geetha SP, Indira B. Micropropagation of *Baliospermum montanum* (Willd.) Muell.-Arg. - A red listed medicinal plant. *J Plant Sci* 2008; **3**(1): 111-5.

[22] Sasikumar S, Raveendar S, Premkumar A, Ignacimuthu S, Agastian P. Micropropagation of *Baliospermum montanum* (Willd.) Muell. Arg. - A threatened medicinal plant. *Indian J Biotechnol* 2009; **8**(2): 223-6.

[23] Singh K, Sudharsana MS, Singh K. *In vitro* propagation of *Baliospermum axillare* Blume. *Indian J Plant Physiol* 2003; **8**: 125-8.

[24] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 1962; **15**: 473.

[25] Jain SC, Jain R, Vlietinck AJ. *In vivo* and *in vitro* antimicrobial efficacy of *Mimosa hamata*. *Indian J Biotech* 2004; **3**: 271-3.

[26] Harborne JB. *Phytochemical methods*. London: Chapman and Hall; 1973.

[27] Victor B, Maridass M, Ramesh U. Antibacterial activity of flower and fruit extracts in *Citrullus lanatus*. *J Econ Physiol* 2002; **5**: 1-3.

[28] Ana Ćirić, Branka Vinterhalter, Katarina Šavikin-Fodulović, Marina Soković, Vinterhalter D. Chemical analysis and antimicrobial activity of methanol extracts of celandine (*Chelidonium majus* L.) plants growing in nature and cultured *in vitro*. *Arch Biol Sci Belgrade* 2008; **60**(1): 7-8.

[29] Johnson M, Maridass M, Irudayaraj V. Preliminary phytochemical and anti-bacterial studies on *Passiflora edulis*. *Ethnobotanical Leaflets* 2008; **12**: 425-432.

[30] Johnson M, Babu A. Somoclonal variation studies on *Passiflora mollussima* (H.B.K.) Bailey using phytochemical methods. *Nat Prod* 2010; **6**(1): 5-10.

[31] Johnson M. Somoclonal variation studies on *Phyllanthus amarus* Schum and Thonn. *Iran J Biotechnol* 2007; **5**(4): 240-5.