International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 10, Issue 9, 2018

Original Article

IN VITRO CALLUS INDUCTION AND COMPARATIVE GC-MS ANALYSIS OF METHANOLIC EXTRACTS OF CALLUS AND LEAF SAMPLES OF *AMPELOCISSUS LATIFOLIA* (ROXB.) PLANCH

DEEP CHHAVI ANAND, RISHIKESH MEENA, VIDYA PATNI

Tissue Culture and Biotechnology Laboratory, Department of Botany, University of Rajasthan, Jaipur 302004
Email: chhavianand03@gmail.com

Received: 10 Jun 2018 Revised and Accepted: 30 Jul 2018

ABSTRACT

Objective: The aim of the present study was to develop a callus induction protocol and comparative study of therapeutic phytochemicals present in *in vivo* leaf and *in vitro* callus extracts through Gas Chromatography-Mass Spectrometry analysis.

Methods: Murashige and Skoog media was used as culture media for callus induction. *In vitro* callus induction protocol was developed by studying the effects of various plant growth regulators like auxin, 2, 4-D (2,4-dichlorophenoxyacetic acid), NAA (naphthalic acetic acid), alone and in combination with cytokinin BAP (benzyl aminopurine), on leaf and stem explants. The GC-MS analysis of *Ampelocissus latifolia* was carried out on Shimadzu QP-2010 plus with thermal desorption system TD 20 to study the phytochemical profile.

Results: *In vitro* callus induction protocol was developed for the plant and callusing was done from leaf and stem explants of *Ampelocissus latifolia*. The best result for callus induction was obtained using leaf explant, and callus production were maximum in Murashige and Skoog medium fortified with BAP (0.5 mg/l) and NAA (1.0 mg/l). Major compounds identified in the GC-MS analysis were Campesterol, Stigmasterol, Beta-Sitosterol, Docosanol, Dodecanoic acid, etc., in *in vitro* extract and Beta Sitosterol, Tocopherol, Squalene, Bergamot oil, Margarinic acid, Hexadecanoic acid, etc., in *in vivo* extract. The different active phytochemicals identified have been found to possess a wide range of biological activities, thus this analysis forms a basis for the biological characterization and importance of the compounds identified for human benefits.

Conclusion: This is the first report on callus induction in *Ampelocissus latifolia*. From the results obtained through the *in vitro* callus induction and its comparative GCMS analysis with *in vivo* extract, it is revealed that *Ampelocissus latifolia* contains various bioactive compounds that are of importance for phytopharmaceutical uses. The GCMS analysis revealed that the amount of Beta-sitosterol and 5-Hydroxymethylfurfural (HMF) was very high in *in vitro* extract as compared to *in vivo* extract.

Keywords: *Ampelocissus latifolia*, GC-MS, Growth regulators, Stigmasterol, Callus, Phytochemicals

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DOI: http://dx.doi.org/10.22159/ijpps.2018v10i9.27879

INTRODUCTION

From ancient times, medicinal plants have been used extensively for their tremendous healing properties and health benefits. India has a treasure of medicinal plants due to the rich diversity in its agro-climatic conditions [1]. The curative properties of medicinal plants are mainly due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, etc. present in them [2]. Plant tissue culture plays an important role in the production of *in vitro* plants and in the manipulation of plants for improved performance. Media composition mainly the hormonal balance is an important factor influencing *in vitro* culture initiation and plant regeneration from explants [3].

The plant *Ampelocissus latifolia* (Roxb.) Planch. belonging to the family Vitaceae, is an example of ethnomedicinal plant species which is the source of natural dye and a range of traditional medicines that cure various diseases[4]. It is a large herbaceous climber, with a tuberous rootstock. The roots have been used for the treatment of snake bite and for its astringent effect. The decoction of the root is also used in chronic dysentry. The tribes of Bihar used this plant for muscular pains, sores and fractured bones [5]. The common name of the plant is "Jungli angoor" and other common name is "Panibel" in India. Juice of tender leaves is used in dental problems and as a detergent for indolent ulcers. The stem ash is applied abdominally for easy delivery in pregnancy [6, 7]. Due to enormous medicinal properties, the plant has been used extensively. Therefore *in vitro* callus from leaves and stem have been raised using various growth regulators like 2, 4-D, NAA alone and in combination with BAP. In recent years, Gas Chromatography-Mass Spectrometry (GC–MS) has been applied unambiguously to identify the structures of different phytoconstituents in plant extracts and biological samples with great success, so further the methanolic leaf and callus extracts were subjected to evaluation for Gas Chromatography-Mass Spectro-photometry analysis to study the phytochemical profile. The unknown organic compounds in a complex mixture can be determined by interpretation and also by matching the spectra with reference spectra [8, 9]. Thus, the phytochemical screening may be useful for the detection and isolation of various bioactive compounds which subsequently may lead to drug discovery and development from these plants *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

Mercuric chloride, bavistin, tween20 detergent were obtained from Sigma–Aldrich. Growth regulators like NAA, BAP, 2,4-D, and methanol were purchased from HiMedia. All chemicals were of analytical grade and used without any further purification. The GC-MS analysis of *Ampelocissus latifolia* was carried out on Shimadzu QP-2010 plus with thermal desorption system TD 20.

Collection and preparation of a plant extract

The plant was collected from Baghdhara Nature Park, Udaipur, Rajasthan. Leaves and stem were collected and used as explants for callus induction.

For *in vitro* callus induction, fresh leaves and stem were collected from the plant and washed under running tap water for 10 min. Then washed with mild detergent tween 20 with gentle and continuous shaking and surface sterilized using bavistin fungicide 0.1% (w/v) for 60 seconds. The leaves were then washed thrice using distilled water. Under Laminar air hood, the explant was surface sterilized using 0.1% (w/v) mercuric chloride solution for 1 minute and then washed thrice with sterile distilled water (1 minute each).

For GC-MS profiling, explants were washed thoroughly with water to remove dirt. They were dried in the shade and ground into powder with the help of a grinder. The dried material (5 grams) was soxhlet extracted using 80% methanol as a solvent for 24 h. Both the extracts were air dried, and finally, weight was taken.

Culture media and tissue culture

Murashige and Skoog media was used as culture media for callus induction. MS media consists of various macronutrients, macro-nutrients, vitamins and organics required for *in vitro* growth of the plant [10] and fortified with 3% (w/v) sucrose as a carbon source and solidified using agar 0.8% (w/v). Various growth regulators like 2, 4-D, NAA alone and in combination with BAP were added, and then the pH of the media was adjusted to 5.8±0.1.

The media was then autoclaved at 121 °C for 15 min at 15 psi pressure. The surface sterilized leaves were cut from edges and then into small explants of size approximately 5 mm^2 and cultured on MS media supplemented with different plant growth regulators. Cultured flasks were incubated in a culture chamber under suitable *in vitro* conditions. The temperature of the chamber was maintained at 25±2 °C using the air conditioner, and light intensity (1200 lux) was provided from fluorescent tubes (40 watt) and incandescent bulbs (40 watts). A photoperiod of 16 h light was provided inside the culture chamber.

RESULTS AND DISCUSSION

Callus induction

In vitro callus induction protocol was developed for the plant and callusing was done from leaf and stem explants of *Ampelocissus latifolia*. The leaves and stem were cut into small segments and used as explants. The effects of the different concentrations of growth regulators on callus formation were determined. These explants were cultured on callus induction medium i.e. Murashige and Skoog medium consisting of different concentrations of different auxins and cytokinin B. A. P. The different concentrations of 2, 4-D (0.5, 1.0, 1.5, 2.0 mg/l), NAA (0.5, 1.0, 1.5, 2.0 mg/l) and in combination 2,4-D+BAP (0.5+1.0, 1.0+1.0, 1.0+0.5, 1.5+1.0 mg/l) and NAA+BAP (0.5+0.5, 0.5+1.0, 1.0+0.5, 1.0+1.0 mg/l) were used. The best result was obtained using leaf explant and callus production was maximum in Murashige and Skoog medium fortified with BAP (0.5 mg/l) and NAA (1.0 mg/l).

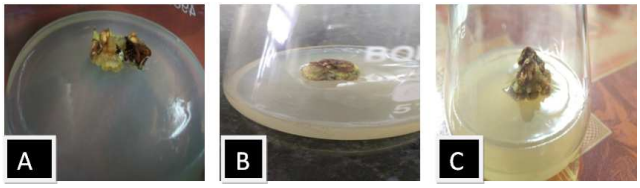

Fig. 1: Callus generated from leaf explant using different growth regulators, A-Callus induction from leaf supplemented with 2, 4-D (0.5 mg/l), B-Callus induction from leaf supplemented with NAA (1.0 mg/l), C-Callus induction from leaf supplemented NAA (1.0 mg/l) and BAP (0.5 mg/l)

Fig. 2: Callus generated from stem explant using different growth regulators, D-Callus induction from stem supplemented with 2, 4-D (1.0 mg/l), E-Callus induction from stem supplemented with NAA (0.5 mg/l), F-Callus induction from stem supplemented NAA (1.0 mg/l) and BAP (0.5 mg/l)

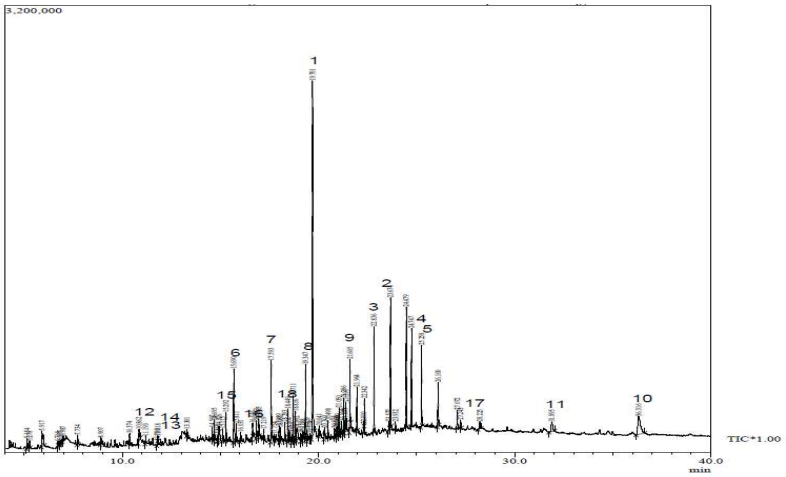

Fig. 3: GCMS chromatogram of methanolic leaf extract of *Ampelocissus latifolia*

Phytochemical analysis by GC-MS

GCMS analysis was done to determine the bioactive compounds from methanolic leaf and callus extracts of *Ampelocissus latifolia*. The analysis of GC-MS chromatogram (fig. 3 and 4) showed peaks of various useful phytochemical constituents present in methanolic extracts of *Ampelocissus latifolia in vitro* and *in vivo*. On comparison of mass spectra of phytoconstituents with the NIST library, various bioactive compounds were characterized and identified (table 1 and 2).

Table 1: Major peaks identified in *in vivo* methanolic extract of *Ampelocissus latifolia*

Peak #	R, Time	Area %	Name
1.	19.701	21.05	n-Hexadecanoic acid
2.	23.674	5.10	Pentacosane
3.	22.836	4.19	Heneicosane
4.	24.747	4.10	Bis(2-ethylhexyl) phthalate
5.	25.258	3.38	Tetracontane
6.	15.690	3.50	Diethyl Phthalate
7.	17.593	3.50	Tetradecanoic acid
8.	19.347	2.91	Hexadecanoic acid, methyl ester
9.	21.605	3.23	Octadecanoic acid
10.	36.316	4.51	Beta-sitosterol
11.	31.895	1.30	Vitamin E
12.	10.862	0.91	5-Hydroxymethylfurfural
13.	11.193	0.32	Bergamot mint oil
14.	11.818	0.22	Ascaridole epoxide
15.	15.292	1.28	Dodecanoic acid
16.	16.031	0.39	Carotol
17.	27.243	0.45	Squalene
18.	18.449	1.14	Neophytadiene
19.	20.498	0.69	Palmitic Acid, TMS derivative

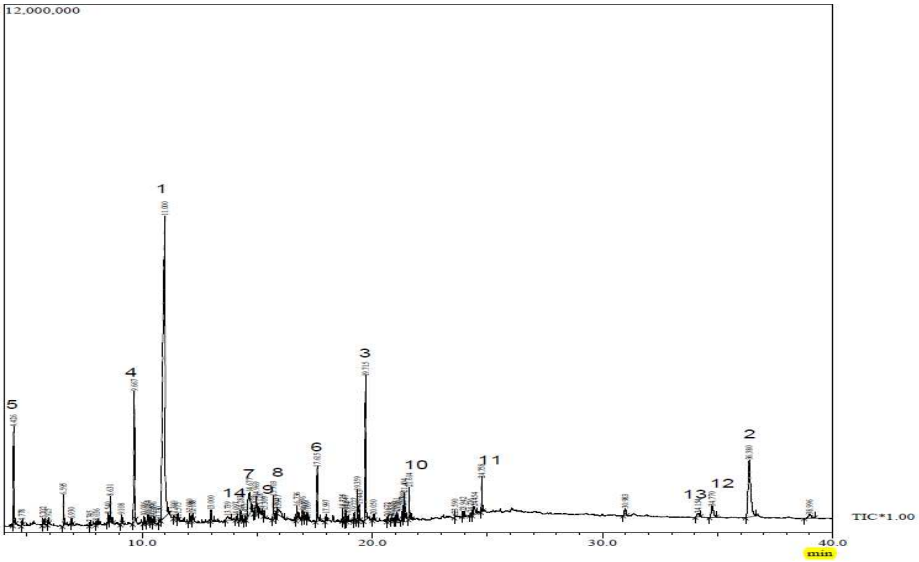

Fig. 4: GCMS chromatogram of methanolic callus extract of *Ampelocissus latifolia*

Table 2: Major peaks identified in *in vitro* methanolic extract of *Ampelocissus latifolia*

Peak#	R. Time	Area%	Name
1.	11.000	42.13	5-Hydroxymethylfurfural (HMF)
2.	36.380	9.19	Beta-Sitosterol
3.	19.715	6.60	n-Hexadecanoic acid
4.	9.667	6.28	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
5.	4.426	3.18	Furfural
6.	17.615	2.22	Tetradecanoic acid
7.	14.677	3.51	D-Allose
8.	15.703	0.94	Diethyl Phthalate
9.	14.969	1.06	Caryophyllene
10.	21.614	1.25	Octadecanoic acid
11.	24.758	1.11	1,2-Benzenedicarboxylic acid
12.	34.770	1.64	Stigmasterol
13.	34.154	0.52	Ergosterol
14.	13.739	0.75	Lactone G
15.	15.309	0.19	Dodecanoic acid
16.	20.050	0.23	Docosyl pentafluoropropionate
17.	20.672	0.14	Margarinic acid
18.	21.297	0.24	Methyl stearate
19.	24.276	0.14	3-Hydroxypropyl palmitate, TMS derivative

Pharmaceutically and economically important phyto-components found in both *in vivo* and *in vitro* extracts and their biological activity

The present investigation was carried out to develop a protocol for in vitro callus induction of Ampelocissus latifolia (Roxb.) Planch. and to determine the bioactive compounds from its methanolic leaf and callus extract through GC-MS. The preliminary phytochemical studies of Ampelocissus latifolia showed the presence of carbohydrates, glycosides, tannins, alkaloids, saponins, flavonoids, steroids, phenols, proteins, hexose sugars, mucilages and gums [11]. Major compounds identified in both the extracts were Campesterol, Beta-sitosterol, Myristic acid, Stigmasterol, Diethyl phthalate, Propanoic acid, Malonic acid, Furyl hydroxymethyl alcohol, Butanedioic acid, Furfural, Linoleic acid, Caproic acid, Dodecanoic acid, Octadecanoic acid, etc. Few compounds were present only in callus extracts like Campesterol, Docosyl pentafluoropropionate, Lactone G, D allose, Dodecanol, Caryophyllene and Tocopherol, Squalene, Bergamol, Neophytadiene, only in leaf extracts. Phytoconstituents with maximum peak area in in vivo extract are Hexadecanoic acid (21.05%), Pentacosane (5.10%), β-sitosterol (4.51%), Tocopherol (1.30%) and 5-Hydroxymethylfurfural (42.13%), β-sitosterol (9.19%), Hexadecanoic acid (6.60%), Stigmasterol (1.64%). All identified compounds generally, were reported to have antimicrobial, antioxidant, anti-inflammatory and anti-cancerous activity and various applications in cosmetic and biofuel industry.

Stigmasterol is a phytosterol, used as a suitable precursor for semi-synthetic progesterone manufacturing [12], as well as a precursor of vitamin D_3 and related compounds [13]. Beta-Sitosterol is also a phytosterol, whose structure is similar to cholesterol and its consumption significantly lowers bad cholesterol levels. Pharmacological screening of β-sitosterol revealed various activities like antimicrobial, anti-inflammatory, anticancer, antifertility, angiogenic, antioxidant, immunomodulatory, antidiabetic, without major toxicity [14, 15]. Campesterol is also a plant-derived sterol having structural similarity with cholesterol, thus competitively inhibits the absorption of cholesterol and may act in cancer prevention [16]. Docosanol is recently approved for pharmaceuticals by FDA (food and drug association) as an antiviral agent for reducing the pain and duration of cold sores caused by Herpes virus by tropical application [17]. Diethyl phthalate is used as Plasticizer. Linoleic acid is used in beauty products as an anti-inflammatory, anti-oxidant, acne reductive, skin lightening and moisture retentive agent on the skin [18]. Dodecanoic acid, Myristic acid, and Octadecanoic acid are used in soaps, and cosmetic industries and Lauric acid is used in the treatment of acne [19]. Hexadecanoic acid has been used in targeting the reduction of cardiovascular disease [20] obesity-related diseases and recently, cancer prevention [21]. Caryophyllene is a sesquiterpene which has anti-tumor, analgesic, antibacterial, anti-inflammatory, sedative, and fungicide activity [22]. Squalene has anti-cancerous properties and used as an adjuvant in vaccines and also in cosmetics [23]. Bergamot mint oil is used as essential oil in Aromatherapy and used for various health benefits [24] and Tocopherol also known as vitamin E has various beneficial effects for skin and human health [25]. Ascaridole epoxide was found to be having strong antimalarial and insecticidal activities [26]. 5-Hydroxymethylfurfural (HMF) has been found to bind specifically with intracellular sickle haemoglobin, for treatment of sickle cell disease [27] and is beneficial to human health by providing anti-oxidative, anti-allergic, anti-inflammatory, anti-hypoxic, and anti-hyperuricemic effects [28] Studies have showed that 5-HMF displayed higher antiproliferative activity on human melanoma A375 cells than other cell lines and could be developed as novel natural antioxidant with potential applications in cancer chemoprevention [29]. Neophytadiene is reported to have antifungal, antipyretic, anti-inflammatory and analgesic properties [30].

CONCLUSION

It is the first report of in vitro callus induction in Ampelocissus latifolia and its comparative GC-MS analysis with that of in vivo leaf extract for identification of individual phytochemicals. From the results obtained through the in vitro callus induction and its comparative GC-MS analysis within vivo extract, it is revealed that Ampelocissus latifolia contains various bioactive compounds that are of importance for phytopharmaceutical uses. 63 different compounds were detected from leaf extract, and 59 phyto-compounds were observed from its callus extract. Few compounds were present only in callus extracts like Campesterol, Stigmasterol, Docosyl pentafluoropropionate, Lactone G, D allose, Dodecanol while Tocopherol, Squalene, Bergamol, were present only in leaf extracts. The GC-MS analysis revealed that the amount of Beta-sitosterol and 5-Hydroxymethylfurfural (HMF) was very high in in vitro extract as compared to in vivo extract. Majority of the common phytoconstituents like Hexadecanoic acid, Dodecanoic acid, Diethyl Pthalate, Octadecanoic acid, etc., were higher in leaf extract as compared to callus extract. Due to the identification of a large number of useful compounds in this species, the plant is considered phytopharmaceutical important for drug discovery and development.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICTS OF INTERESTS

Declared none

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