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ANTIMICROBIAL ACTIVITY OF SAFED MUSLI (*CHLOROPHYTUM BORIVILIANUM* L.) CALLUS EXTRACT AND GC-MS BASED CHEMICAL PROFILING

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Abstract

An efficient protocol was established to induce Safed musli (*Chlorophytum borivilianum* L.) callus using shoot bud explants inoculated on MS media supplemented with various concentrations of 2,4-D, NAA and BA. The highest callus induction frequency (71.6) and mean fresh weight (12.0 ± 1.1 g) was evidenced on media containing 5 mg/l 2,4-D. The next best treatment was found to be 2 mg/l NAA and 1 mg/l BA supplemented media with 57.3% callus induction frequency and fresh weight of 7.2 ±1.2 G. There was a significant variation in callus morphology influenced by different plant growth regulators. The callus extract effectively inhibited all the tested microbes. The highest zone of inhibition was observed against *Candida albicans* (12.7 ± 0.6 mm), followed by *Pseudomonas aeruginosa* (11.6 ± 0.6 mm), *Bacillus subtilis* (09.0 ± 1.0 mm) and *Staphyloccus aureus* (7.3 ± 1.1 mm) at 300 µg concentration. GC-MS analysis of the callus extract revealed the occurrence of 16 bioactive compounds including Butana l, 3-methyl-, cyclohexanamine, cyclopropylamine, 2,3-butanediol, [R-(R*,R*)]-, 1-propanamine, 2-methyl-N-(2-methylpro-pylidene and n-hexadecanoic acid. Therefore, Safed musli callus can be used as an alternative potential source of secondary metabolites for therapeutical applications.

Introduction

Chlorophytum borivilianum L. commonly known as 'Safed musli' is an important medicinal plant. The plant is valued for its tuberous roots which contain abundant bioactive constituents, such as saponins, phenols, flavonoids, alkaloids, steroids, tannins, triterpenoids, and vitamins (Nakasha *et al.* 2016a). The bioactive metabolites of Safed musli possess higher aphrodisiac activity and hence, widely employed for treating several sexual problems. In addition, the plant is useful in curing diabetes, chronic leucorrhoea, high blood pressure, arthritis and delayed menopause (Khanam *et al.* 2013). It is a part of various traditional medical practices Ayurveda, Unani, Homeopathy and Allopathy. Commercial cultivation of Safed musli is hindered due to seed dormancy and low germination. As an alternative approach, tubers are used as planting materials but increase the cost of cultivation because tubers are expensive. In addition, tubers show dormancy and asynchronous germination making difficulty in crop management in the field (Nakasha *et al.* 2016b). Plant cell and tissue culture could be an effective approach for producing bioactive metabolites of Safed musli under controlled environment. It is also stated that plant cell cultures result higher biomass with relatively higher bioactive products (Kumar *et al.* 2014). Plant growth regulators are known to influence on cell growth, morphogenesis, and secondary metabolite accumulation (Mohanty *et al.* 2014). It has been proved that callus culture as a reliable source for plant secondary metabolites (Hussain *et al.* 2012, Kumar *et al.* 2014, Nakasha *et al.* 2016b).

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The use of an optimal culture conditions promotes the production of secondary metabolites in any plant species. Recent years have witnessed the increase of multi-drug resistant microbes around the globe and is a great threat. Hence, scientists are forced to investigate and discover novel effective antimicrobial agents against these drug resistant pathogens (Rudramurthy *et al.* 2016). Researchers have established that callus extract of different plant species possesses various biological activities including antioxidant, antimicrobial and cytotoxic activities (Landa *et al.* 2006, Johnson *et al.* 2011, Kumar *et al.* 2014). Further, GC-MS analysis of callus extracts was successfully employed for identifying various bioactive metabolites (Ali and Tariq 2013, Cheong *et al.* 2016). Nakasha *et al.* (2016b) have proposed callus induction protocol for Safed musli however, an efficient protocol is yet to be established. To date, no reports are available on the secondary metabolites of Safed musli callus extracts and their biological properties. Therefore, the present study was undertaken to establish a simple reliable protocol for inducing callus in Safed musli. Furthermore, callus extract was evaluated for antimicrobial activity against human pathogens and the phytocompounds were identified by using GC-MS analysis.

Materials and Methods

Tubers of Safed musli harvested from the field were kept for maturation under dark condition at 28°C for one month. About 1 - 2 cm long shoots (explants) sprouted from the tubers were carefully washed under running tap water and immersed in bavistin (fungicide) solution (5 g/l) for 2 hrs. Explants were washed two times by using sterilized distilled water and immersed in 0.1% NaOCl solution containing 2 - 3 drops of Tween-20 for 20 min. Later, explants were taken to laminar air flow and treated with $HgCl_2$ solution (0.1%) for 15 min and rinsed three times in sterilized distilled water. Later, these shoot buds were vertically incised and implanted on solid MS medium supplemented with different plant growth regulators to induce callus formation. The culture media used were as follows: MS + 3 mg/l 2,4-D, MS + 5 mg/l 2,4-D, MS + 2 mg/l NAA + 0.5 mg/l BA and MS + 2 mg/l NAA + 0.5 mg/l BA, MS + 2 mg/l NAA + 1.0 mg/l BA and MS + 2 mg/l NAA + 1.5 mg/l BA. All media were adjusted to pH 5.7 ± 0.1 prior to the addition of 0.8% agar and autoclaved at 121ºC, and 15 lbs pressure for 20 min. All inoculated culture bottles after inoculation were incubated in a clean growth chamber at 25 ± 1°C under a 16/8 hrs (light/dark) photoperiod with a light intensity of 3000 lux supplied by cool-white fluorescent lamps and relative humidity of over 70%. The data on callus formation frequency (%), mean fresh weight (g), callus growth and morphology were recorded after 60 days of culture. The best callus formed on the media was harvested and dried in an oven at 60^0C until a constant weight was obtained. The dried callus was finely powdered using mortar and pestle and added into 80% methanol (1 : 10), and continuously agitated (100 rpm) on a rotary shaker for 24 hrs in the culture room (25°C). The mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was evaporated to dryness using rota-vapor. The antimicrobial activity of Safed musli callus extract was tested against *Bacillus subtilis* B29, Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC700698) (Gram-positive), *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia coli* E266 (Gram-negative) and *Candida albicans* 90028. The experiment was performed by using disc diffusion method as described by Kumara *et al.* (2012). In short, 10 mg of the callus extract was dissolved in 1 ml of dimethyl sulfoxide (DMSO) and about 10, 20 and 30 µl of this solution was impregnated on 6 mm sterilized Whatman No.1 filter paper discs. Later, the air-dried discs were placed on culture plates pre-inoculated with known microbial strains. The discs saturated with DMSO (20 µl) served as a negative control for all microbes while, streptomycin (100 mg/ml) and Nystatin (100 mg/ml) served as a positive control for bacterial and fungal species, respectively. All microbes were incubated in an incubator at 37ºC for 24 hrs to observe for the zone of growth inhibition (mm) around each disc. For each microbial strain, the experiment was repeated in

triplicate. GC-MS analysis using the model instrument, GCMS-QP2010 Ultra (Shimadzu Co., Japan) attached with a capillary column DB-1 (0.25 µm film × 0. 25 mm I. d. × 30 m length). Analysis was performed by injecting 1 µl of callus extract (dissolved in HPLC grade methanol) with a split ratio of 20 : 1. Helium gas (99.9%) was used as the carrier gas at a flow rate of 1 ml/min. The analysis was performed in the EI (electron impact) mode with 70 eV of ionization energy. The injector temperature was maintained at 250°C (constant). The column oven temperature was set at 50°C for 3 min, raised at 10°C per min to 280°C for 3 min and finally held at 300°C for 10 min. The compounds were identified after comparing the spectral configurations obtained with that of available mass spectral database (NIST and WILEY libraries). In each experiment, the data recorded was from 3 replications (n = 3) and all the results are represented as mean ± Sd. One-way ANOVA was carried out to compare the data. Further, to determine the statistically significant differences, the Tukey's test was performed at $p < 0.05$ level using Graph Pad Prism (version 5.0) statistical software.

Results and Discussion

The influence of plant growth regulators (2,4-D, NAA and BA) on callus induction from shoot bud explants of Safed musli was studied. Callus formation was triggered in all the media (Table 1). However, the morphogenetic pattern varied significantly depending on the type of plant growth hormones supplemented in the media. Many authors have commonly used 2,4-D, NAA and BA in the culture media for inducing callus in many plant species (Kumar *et al.* 2014).

Table 1. Effect of plant growth regulators on callus induction of *C. borivilianum* explants after 60 days of culture.

MS + growth regulators (mg/l)			Callus formation (%)	Mean weight of callus (g)	Degree of response	Callus morphology (color/texture)
2, 4-D	NAA	BA				
0	0	0	-	-	-	-
3	0	0	45.7 ± 1.1 c	6.5 ± 0.7 c	+++	Yellowish/friable
5	0	0	71.6 ± 1.5 a	12.0 ± 1.1a	++++	Yellowish/friable
0	2	0.5	24.7 ± 1.5 d	1.7 ± 0.6 d	+	White/compact
0	2	1	42.7 ± 1.5 c	4.8 ± 0.6 c	++	Light green/compact
0	2	1.5	57.3 ± 2.0 b	7.2 ±1.2 bc	+++	Light green/compact

Each value is expressed as mean ± standard deviation (Sd) (p = 10). Values in the column followed by a different letter superscript are significantly different ($p < 0.05$). The symbol "+++" and "++++" indicate that the degree of response is good and excellent, respectively.

In the present study, 5 mg/l 2,4-D supplemented media resulted the highest callus formation frequency (71.6 ± 1.5%) and callus fresh weight (12.0 ± 1.1 g). The callus was yellowish in color and the texture was found to be friable in nature. The degree of callus formation was excellent on the same media. While, 2 mg/l NAA along with 1.5 mg/l BA induced 57.3 ± 2.0% callus formation frequency and the fresh weight was observed to be 7.2 ± 1.2 g. However, calli were morphologically greenish and compact in nature. Our results support the findings of Nakasha *et al.* (2016a), where 5 mg/l was effective for callus formation in Safed musli shoot bud explants. Several researchers have mentioned that 2,4-D as the best plant growth regulator for inducing callus in many plant species (Rathore *et al.* 2011, Johnson *et al.* 2011, Sen *et al.* 2014). It has been stated that callus induction and growth are stimulated by the synergistic effect of NAA and BA when used in combinations (Kumar *et al.* 2014). The synergic effect of hormones has induced

green colored compact callus at higher levels of BA. Similar observations were made by Kumar and Nandi (2015) in *Asteracantha longifolia*. Callus formation was completely absent in growth regulator free media.

The antimicrobial potential of Safed musli callus extract is shown in Table 2. It is evident from the result that all microbial species evaluated were inhibited by the callus extract at all concentrations. However, the degree of inhibition differed from one to another. It was also noticed that a higher antimicrobial activity was observed with the increased concentration. *C. albicans* and *P. aeruginosa* ATCC 15442 were found to be more susceptible to the extract with the highest inhibitory activity of 11.6 ± 0.6 mm and 12.7±0.6 mm, respectively at the concentration of 300 µg/disc. The lowest activity (04.0 ± 1.0 mm) was observed in *S. aureus* (MRSA) at lower concentration (100 µg/disc) and at higher concentration (300 µg/disc) it increased to 7.3 ± 1.1 mm. It has been reported that callus extract of calli of *Nigella* species significantly inhibited *S. aureus*, *Bacillus cereus* and *Staphylococcus epidermidis* (Landa *et al.* 2006). Likewise, callus extract of *Mentha arvensis* have shown inhibitory potential against *Salmonella typhi, Streptococcus pyogenes, Proteus vulgaris* and *Bacillus subtilis* (Johnson *et al.* 2011). Also, it was noticed that *in vitro* raised calli extract exhibited superior activity than the *in vivo* tissue extract. Likewise, our results are in support of previous observations on *Baliospermum axillare, Passiflora edulis, Rauvolfia tetraphylla, Physalis minima* callus extracts against several microbial pathogens (Singh and Sudharshana 2003, Shariff *et al.* 2006, Johnson *et al.* 2011). This indicates that callus extract of Safed musli possesses broad spectrum bioactive compounds and could serve as a novel source for antimicrobial agents.

Table 2. Antibacterial activity of *C. borivilianum* callus extract (methanolic) against human pathogens at different concentrations.

Solvent extracts (µg/disc)	Zone of inhibition* (mm)				
	Bacillus subtilis B29	*Staphyloccus aureus* (MRSA)**	*Pseudomonas aeruginosa* ATCC 15442	*Escherichia coli* E266	*Candida albicans* 90028
100	06.3 ± 0.5	04.0 ± 1.0	08.3 ± 1.5	04.0 ± 1.0	07.3 ± 2.0
200	08.3 ± 1.5	05.6 ± 0.6	10.0 ± 1.7	06.0 ± 1.0	12.0 ± 1.0
300	09.0 ± 1.0	7.3 ± 1.1	11.6 ± 0.6	07.6 ± 0.5	12.7 ± 0.6

*The experiment included DMSO (20 µl) as negative control while, streptomycin (100 mg/ml) for bacteria and nystatin (100 mg/ml) for yeast served as positive control. Each value represents the mean ± standard deviation (Sd) of 3 replicates per treatment in 3 repeated experiments. ** MR represents methicillin resistant.

The presence of various secondary metabolites in the callus extract of Safed musli was analyzed by using GC-MS chemical profiling and the results are presented in Table 3. A total of 16 compounds with 6 identified and 10 unidentified compounds were determined in the extract (Fig. 1). The identified compounds included butanal, 3-methyl-, cyclohexanamine, cyclopropylamine, 2,3-butanediol, [R-(R*,R*)]-, 1-propanamine, 2-methyl-N-(2-methylpropylidene and n-hexadecanoic acid. Some of these compounds are known to possess various biological activities including antioxidant and antimicrobial activities (Kalaivani *et al.* 2012, Rahman *et al.* 2014).

All the identified compounds were in trace amount and hence, antimicrobial activity observed in the present study could be attributed to these unidentified new compounds. Hence, further exploration of Safed musli callus extract is required for better understanding on the occurrence of bioactive compounds. Similarly, GC-MS analysis has proved the occurrence of various

phytochemicals in different *in vitro* derived plant callus extracts (Ali and Tariq 2013, Cheong *et al.* 2016).

Table 3. GC-MS profile of *C. borivilianum* callus extract (methanolic).

Sl. No.	Name of the compound	Peak no.	R. time	Peak area (%)
1	Butanal, 3-methyl-	1	2.643	0.531
2	Cyclohexanamine	2	2.810	0.577
3	"	3	2.928	0.755
4	Unidentified	4	3.59	1.196
5	"	5	3.594	1.126
6	2,3-butanediol, [R-(R*,R*)]-	6	4.394	0.469
7	1-propanamine, 2-methyl-N-(2-methylpropylidene	7	5.069	0.459
8	Unidentified	8	5.357	0.494
9	"	9	5.544	0.392
10	"	10	20.086	0.568
11	"	11	39.419	17.391
12	"	12	40.383	0.621
13	n-hexadecanoic acid	13	51.424	1.664
14	Unidentified	14	72.571	69.253
15	"	15	86.242	2.768
16	"	16	87.932	1.735

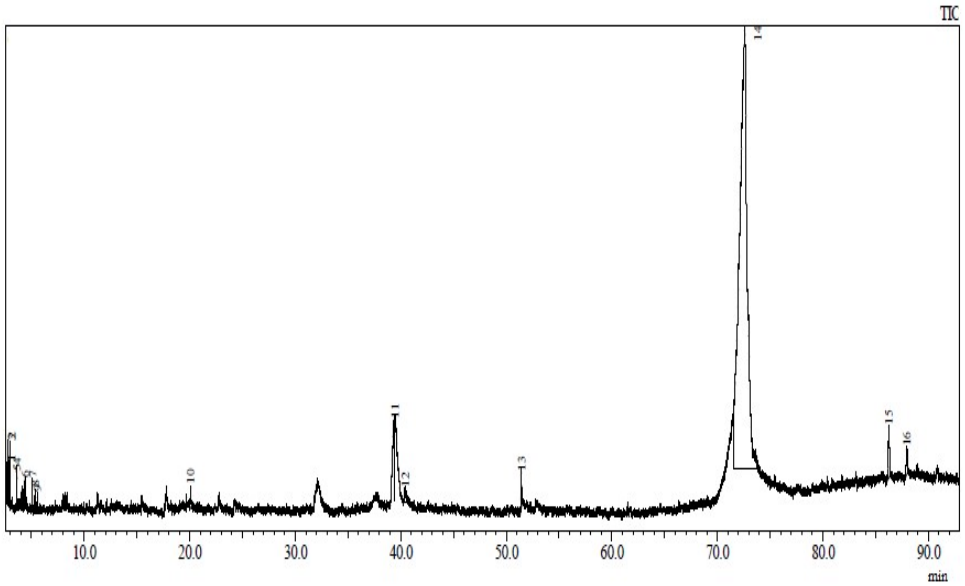

Fig. 1. GC-MS chemical profile of the methanolic callus extract of Safed musli.

In conclusion, callus was better induced on 5 mg/l supplemented MS media. The extract of callus possessed potential antimicrobial activity against various human pathogens and comprised of several secondary metabolites. Thus, Safed musli callus can serve as a new source for secondary metabolites in future for developing novel therapeutically active drugs and evidently deserves further investigations.

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