ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

Volume 10 Issue 1 January 2016

www.abap.co.in

Current Trends in Biotechnology and Pharmacy
ISSN 0973-8916 (Print), 2230-7303 (Online)

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ISSN 0973-8916

Current Trends in Biotechnology and Pharmacy

(An International Scientific Journal)

Volume 10 Issue 1 January 2016

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Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

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Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Current Trends in Biotechnology and Pharmacy

ISSN 0973-8916

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Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) 5-12 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Genetic Polymorphism of *Tylophora* Species of Goa as Revealed by RAPD

Uma Masur[1*], P. Hima Kumari[2], Kailas Gokhale[1] and U.M.X. Sangodkar[3]
[1]Parvatibai Chowgule College of Arts and Science, Gogol, Margao-Goa-403602, India
[2]Department of Genetics, Osmania University, Hyderabad-500 007, India
[3]Department of Marine Biotechnology, Goa University, Bambolim, Panajim, Goa, India
*For Correspondence - umapatil20@yahoo.co.in

Abstract

Tylophora indica is an important medicinal plant and is used against dysentery, diarrhoea, humoral and bronchial asthma, allergic rhinitis, cold and cough. The plant is also known for anticancer properties. Other species of *Tylophora* predominantly found in Goa is *T. dalzelli*. It has been reported as a local herb for the treatment of asthma. Hence the main objective of this study is to know the genetic relationship in these two species. To achieve this, randomly amplified polymorphic DNA (RAPD) was performed. A total of six *Tylophora* variants were collected from various regions of Goa and analyzed using RAPD with OPF 1-10 primers. Genetic similarities were determined between and within the collected species through Jaccard's coefficient system. RAPD primers generated 39 amplification products, of which all 39 were polymorphic. Results from cluster analysis and linkage distance, indicate 38% genetic similarity at the interspecifc level between *T. indica* whereas 10% between two *T. dalzelli* variants. UPGMA cluster analysis revealed similar results.Our study indicated that *T. indica* collected from Valpoi was the most diverse one among all the samples.These results based on the molecular markers have shown high genetic diversity among thecollected species.

Keywords: *Tylophora indica*, *Tylophora dalzelli*, RAPD, medicinal plant, genetic polymorphism.

Introduction

Tylophora indica belongs to the family Asclepiadaceae. It consists of 130 genera and 2000 species. *T. indica* was studied extensively for its antiasthmatic and allergic rhinitis problems (1). *Tylophora indica* is one of the herbs, and mostly used against asthma. Pharmacological investigations have also confirmed the anti-asthmatic properties of its leaf extract (1). The major alkaloid tylophorine has been reported to have immunosuppressive, anti-inflammatory effect (2). The leaf and stem extract having tylophoridine are known to possess anti-leukemic properties also (3). Thus, the plant has been reported to be anti-asthmatic, anti-allergic, hepato protective and immuno modulator (4-5). Significant activity of the extracts of stem and leaf were observed against two standard transplantable tumors, lymphoid leukemia L1210 and lymhocytic leukemiaP388 (6). Hence, there is already a concern about over exploitation and decline in the wild population of *T. indica* species (7). A closely related species is *T. dalzelli*, which is commonly found as a roadside weed. *T. dalzelli* has been reported to be used as a local herb for the treatment of asthma in Goa (8,12). *T. dalzelli* with more or less has same pharmacological properties but the genetic relationship of these two species is not known.

DNA-based molecular markers have been proposed as an excellent approach for identifying geographical variation, genetic diversity, phylogenetic relationship, authentication of plant

Vol. 10 (1) 5-12 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

species, pharmacognostic characterization, species characterization and genetic mapping in medicinal plants (9). Accordingly, the RAPD work carried out on three different species belonging to the family Asclepiadaceae for comparative analysis gave an important clue about the genetic diversity as well as close affinities (10). Similarly, RAPD and ISSRcarried out on *in vitro* generated microshoot through somatic embryogenesis of *Tylophora* showed genomic bands like that of the mother plant signifying uniformity and resemblance to the mother plant (11). Though pharmacognostic studies on *T. dalzelli* were carried out (13) there was no literature on the comparative analysis of secondary metabolites present and their use to treat asthma and other diseases.In the present study, random amplified polymorphic DNA (RAPD) was performed on *T. indica* and *T. dalzelli* species to know their genetic diversity and to determine whether *T. dalzelli* could be also used as a medicinal plant for treating asthma.

Materials and Methods

Plant collection: Six species of *Tylophora* used in this study were collected from five different populations belonging to five different geographical areas of North Goa (NG) and South of Goa (SG), like Pernem (P), Rivona (R), Valpoi (V), Nuvem (C) and Margao (M) (Table 1) and were grown ex-situ. Geographical data of the collection points was identified (Table 1) (14). The plants were brought to the lab andidentified with the help ofThe Flora of the Presidency of Bombay and Flora of Goa, Diu, Daman, Dadra and Nagarhaveli (15- 16). They were also confirmed at the Botany Department, Goa University, Goa. Leaves were harvested after one month and transported to the Plant Genetics Laboratory, Department of Genetics, Osmania University, Hyderabad, for DNA isolation and RAPD analysis.

Isolation of genomic DNA:The plant genomic DNA was isolated according to the protocol of Doyle and Doyle (1987) (17). About 1 g of young leaf tissue was homogenized in liquid nitrogen and the powder was mixed in 5 ml of pre-heated (65^0C) CTAB buffer containing 1% polyvinyl pyrrolidine (PVP) and 0.2% â-mercaptoethanol in 50 ml capacity polypropylene tubes and incubated at 65 ^{0}C in a water bath for 1 h with occasional, gentle swirling. Later, equal volumes of chloroform: isoamyl alcohol (24:1) was added to the samples. The tubes were gently shaken by inverting few times. Centrifugation was carried out at 10,000 rpm for 5 min and the aqueous phase was carefully transferred into a fresh centrifuge tube and mixed well with 0.6 volumes of ice coldisopropanol by gently inverting the tube. This mixture was incubated at -20 ^{0}C for 1 h to allow complete precipitation of DNA and then centrifuged at 12,000 rpm for 10 min to pellet the DNA. The pellet was washed with wash buffer (70% v/v ethanol) and air-dried. Finally, the DNA was dissolved in 100 µl of TE buffer. The quality and concentration of extracted DNA was determined by measuring the absorbance at 260 nm and 280 nm using UV visible spectrophotometer (Thermo scientific). Isolated genomic DNA was run on 1% agarose gel after treatment with RNase A for determining the quality and quantity of DNA.

PCR and RAPD analysis: RAPD was performed for the amplification of target genomic DNA fragments. A total volume of 25 ìl of PCR mix was prepared in a sterile 0.2 ml eppendorf tube with 10 pmol/ìl each of both forward and reverse primers (Table 2), 1 ìl of DNA as a template, 50 µM of each dNTP, 1.5 mM $MgCl_2$ and 1 U of TaqDNA Polymerase (Bangalore Genei). Each PCR aliquot was mixed and the PCR reactions were performed. The standard reaction conditions carried out are given in Table 3. An aliquot from the amplified PCR product was used to analyze on 1% agarose gel and to check the amplification.

Data analysis: RAPD generated DNA banding patterns were converted to binary matrix by assigning 1 for presence of band and 0 for its absence by using multivariate analysis program NTSYS-PC (18). Genetic similarity was calculated with the help of Jaccard's coefficient

of similarity and a phylogenetic dendrogram was constructed by using unweighted pair group with arithmetic mean (UPGMA) taking the help of R-program.

Results

***Plant collection*:** Collected plants from different regions of Goa were identified as *T. indica* and *T. dalzelli*. *Tylophoraindica* (Burm.f.)Merr.Or Indian Ipecac commonly known as Antamul (Marathi), Pitvel (Konkani) is aperennial, woody liana, terrestrial and mesophytic in habitat. *Tylophora dalzellii* Hook.f. known as Dalzell Ipecac, commonly known as Lhan Pitmari in Marathi. It is a twine with terrestrial and mesophyte habitat. Out of six variants collected, four were identified as *Tylophora indica* obtained from Pernem, Margao,Valpoi and Rivona whereas two of them as *Tylophora dalzelli* collected from Rivona and Nuvem. Morphological variations were seen within *Tylophora indica* species obtained from Valpoi, with considerable differences in leaf morphology. They were labeled as *T. indica*

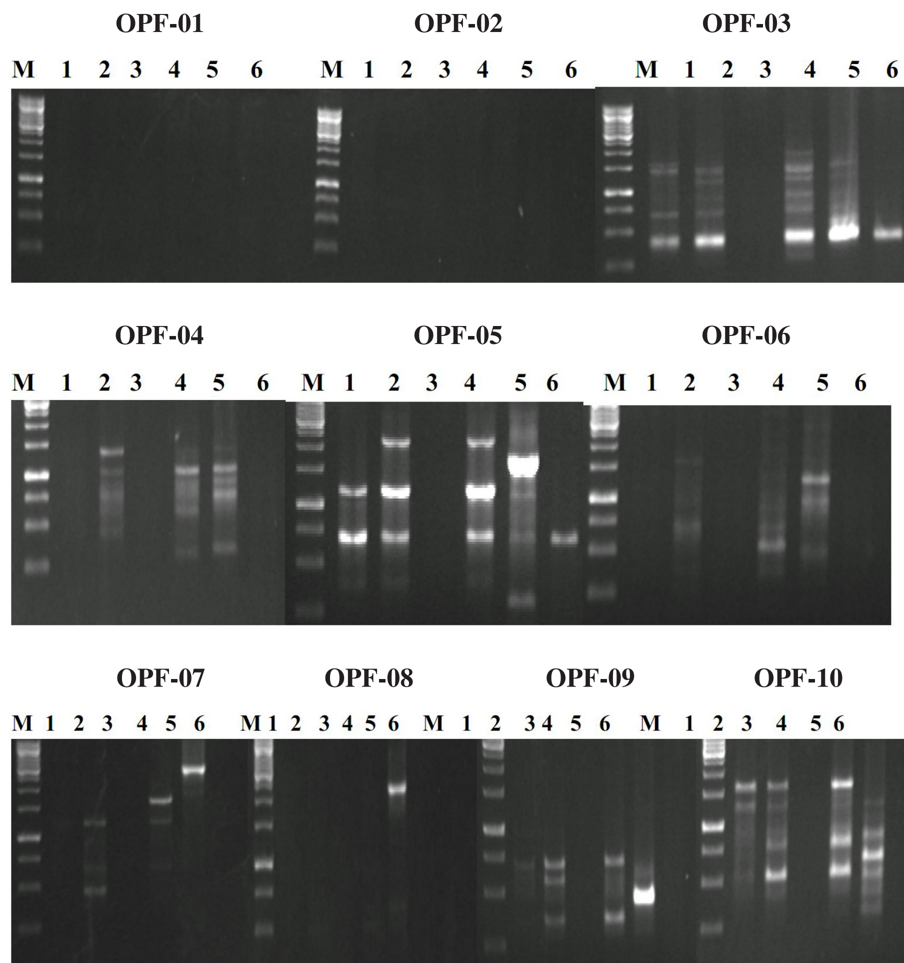

Fig. 1. RAPD PCR with OPF 1-10 primers. M = 1Kb DNA molecular weight marker, Lane 1 = TiP, Lane 2 = TiR, Lane 3 = TiV, Lane 4 = TiM, Lane 5 = TdR,Lane 6 = TdN.

(TiP); *T. indica* (TiM); *T. indica* (TiV); *T. indica* (TiR); *T. dalzelli* (TdR); *T. dalzelli* (TdN).

***PCR and RAPD analysis*:** Quality of genomic DNA was checked using UV-Visible spectrophotometer at 260/280 nm and electrophoresed on 1%agarose gel. Using genomic DNA as a template, amplification was carried out with different RAPD primers of OPF series. Amplified products were confirmed by running them on 1% agarose in 0.5X TAE at constant voltage. Out of 10 OPF primers, DNA of all the *Tylophora* variants was amplified with 8 OPF primers except TiV. OPF-1 and OPF-2 have not shown any bands; whereas OPF-3 to OPF-10 haveexhibited 39 bands varying from 40 to 3000 bp. On an average, each primer has generated 4.875 bands. However, OPF-10 has generated a maximum of 8 bands ranging from 350-1600 bp and OPF-8 has given minimum 1 band of 2700 bp (Table 3; Fig. 1). It is also observed that 100% bands generated by these primers are polymorphic. Similarity indices were developed on the basis of amplified products of 10 RAPD primers with the six variants (Table 4). Genetic similarities calculated using Jaccard's coefficient ranged from 0 to 0.40, its mean and standard deviation (SD) has been found to be 0.28 and 0.34 respectively. UPGMA cluster analysis for the study revealed similar results as that of similarity coefficients with TiM and TiR exhibiting 40% similarity. Variant TiV was the most diverse one among all the samples of *Tylophora*. UPGMA cluster analysis revealed a clear picture (Fig. 2) of all the samples.

Discussion

Molecular screening has become the mandatory technique for screening species/ variants to study their genetic differences, determine authenticity, and to find out phylogenetic relationship of many plants (19) differing in their geographical location. It is possible to resolve the genetic polymorphism if any among different species and variants using RAPD technique. Earlier,genetic diversity analysis among 15 barley landraces was carried out using the same technique (20). Also, cultivars of Tunisian pomegranate were investigated for genetic diversity using universal primers by RAPD (21). The technique is also used to resolve issues regarding medicinal plants. Cluster

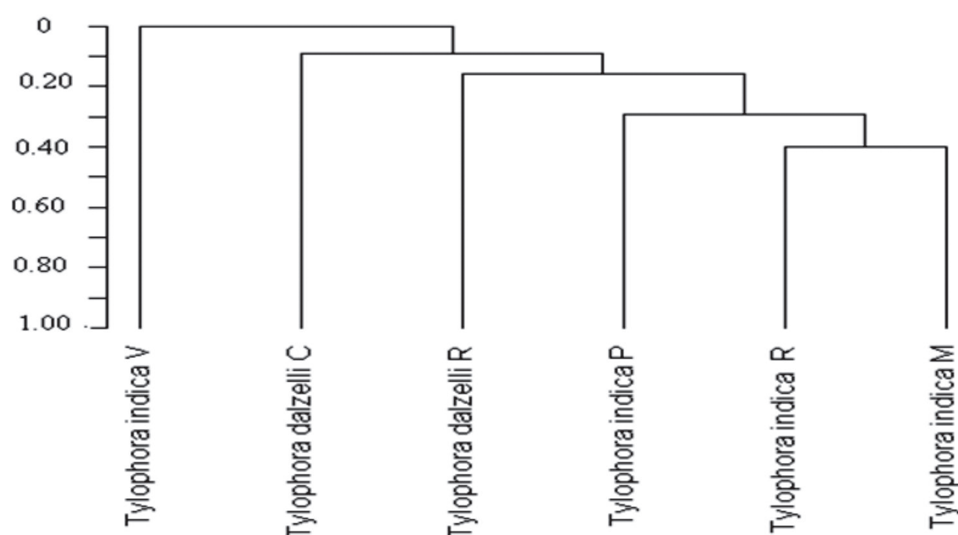

Fig. 2. UPGMA based dendrogram with genetic relationships among the six variants of Tylophora.

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Table 1: Sampling sites of *Tylophora* species in Goa. NG- North Goa, SG- South Goa

Place	Sea level (m)	Geographical Location
Pernem (NG)-TiP	18	15.7169° N, 73.7978° E
Valpoi (NG)-TiV	42	15.5300° N, 74.1300° E
Rivona (SG)-TiR, TdR	31	15.1638° N, 74.1028° E
Nuvem (SG)-TdN	15	15.3089° N, 73.9461° E
Margao (SG)-TiM	31	15.2736° N, 73.9581° E

Table 2: RAPD PCR program conditions

Step	RAPD	
	Temp (^{0}C)	Time
Initial denaturation	94	5 min
Denaturation	94	1 min
Annealing	37	1 min
Elongation	72	2 min
Final Extension	72	10 min
Holding temperature	25	5 min

Table 3: RAPD analysis of *Tylophora* species and variants from Goa

Primer name	Primer sequence	Size of fragments	Number of bands	Polymorphic bands	Polymorphism %
OPF-1	ACG GAT CCT G	-	-	-	-
OPF-2	GAG GAT CCC T	-	-	-	-
OPF-3	CCT GAT CAC C	400-1700	7	7	100
OPF-4	GGT GAT CAG G	350-1900	6	6	100
OPF-5	CCG AAT TCC C	650-2300	5	5	100
OPF-6	GGG AAT TCG G	500-1400	2	2	100
OPF-7	CCG ATA TCC C	400-3000	6	6	100
OPF-8	GGG ATA TCG G	2700	1	1	100
OPF-9	CCA AGC TTC C	40-650	4	4	100
OPF-10	GGA AGC TTG G	350-1600	8	8	100
		Total	39	39	100

Table 4: Relationship among six variants of *Tylophora* as per Jaccard's similarity coefficient

TiP	TiR	TiV	TiM	TdR	TdN	
TiP	1					
TiR	0.38	1				
TiV	0	0	1			
TiM	0.29	0.40	0	1		
TdR	0.16	0.18	0	0.28	1	
TdN	0.22	0.1	0	0.09	0.10	1

analyses of hypericin content and RAPD markers grouped the clones of *Hypericum perforatum* L. in two major clusters and significant correlations were observed between them (22). *Tylophora indica* known for its exemplary medicinal uses faces exploitation and decline in wild populations (7). This also gives scope to use other related plants as adulterants in Ayurvedic preparations, which brings down the quality of drug and demand of herbal drugs in their natural form.

In the present investigation, two species of *Tylophora* known locally for treatment of asthma were used. RAPD was performed to assess the genetic polymorphism among the variants collected. The results showed that out of 10 primers used for amplification, 8 primers showed polymorphic nature of the variants. It was also seen that both the species showed 100% polymorphism. On the other hand, *T. rotundifolia* showed only 46% of polymorphism (23). Results from cluster analysis and linkage distance, indicated that the variant TiV is different from others used in this study. It appears that this plant has been introduced into the nurseries of Goa, but not a native to this place. Also, similarity between TdR and TdN was found to be only 10%. This gives scope to question the factors responsible for such a level of dissimilarity among the variants and species in such a close ecological zone of Goa, being only 3,702 square kilometers. Based on the molecular markers, it is inferred that the two species and the morphological variants collected from different locations are highly variable. This can be attributed to the area, size of species and higher cross pollination among them. A high genetic diversity further supports their diverse origin (24-25). Narrowing of gene pool and reduced genetic diversity pose challenges in the selection pressure brought in by environmental changes (23). The high genetic diversity observed in *Tylophora* species here may be reflecting that the species has adapted well to the environmental conditions (26). But, usage of *Tylophora dalzelli* as an antiasthamatic plant as a replacement of *T. indica* needs further studies through bioassays.

Acknowledgements

Authors wish to thank Prof. P. B. Kavi Kishor, Department of Genetics, Osmania University, Hyderabad, for providing laboratory facilities and guidance to carry out this research work.

Conflict of interest : The authors declare no financial or commercial conflict of interest.

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Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) 13-19 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Optimization and Stability studies of siRNA for Significant Inhibition of Dengue (DENV-2) Viral Replication in Vero cells

Anil Babu Korrapati[1,2*] and Muvva Vijayalakshmi[2]
[1] Recombinant Gene Products Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
[2] Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar-522510, Guntur, Andhra Pradesh, India.
*For Correspondence - anilbabu.korrapati@gmail.com

Abstract

RNA interference (RNAi) is a highly conserved, specific and selective biological response of gene regulation mechanism and has been widely exploited for designing novel drug targets to treat infections caused by pathogens including viruses. In the previous study, one of the promising sh-5b RNA (short hairpin RNA, sh-5b) construct was targeted to the conserved sequence within the 5¹-NTRs (non-translated regions) of all Dengue serotypes. The current study report the stability (up to 24 months) of recombinant adenovirus (rAd) engineered to express sh-5b RNA for DENV-2 replication inhibition. Stability study was done by gene specific PCR, where as the effect of sh-5b RNA in attenuating DENV-2 replication was further validated by One step RT-PCR by TITAN. The rAds harboring sh-5b RNA to target dengue serotypes might serve as an efficient alternative to conventional drugs for therapeutic intervention to treat DENV infections in humans.

Keywords: RNA interference, Dengue, short hairpin RNA, non-translated region, One step RT-PCR, gene specific PCR

Highlights

1. Selection (optimization) and stability (up to 24 months) studies of siRNA (sh-5b RNA) for replication inhibition of DENV-2.
2. Re-confirming the effectiveness of sh-5b (after 24 months) in inhibiting DENV replication by using one step RT PCR by using TITAN enzyme.

Introduction

Dengue fever (DF) is caused by serologically related dengue virus 1, 2, 3 and 4 and is transmitted between people by the mosquitoes *Aedesaegypti* and *A. albopictus.* In some cases, this mild DF may lead to more severe clinical manifestations such as dengue hemorraghic fever (DHF) and dengue shock syndrome (DSS). Despite considerable advances in the development of tetravalent vaccine and antiviral drugs there is no availability of clinically approved vaccine or drug to combat DENV infections. The discovery of the envelope dimer epitope (EDE) and its neutralization by human mAbs might pave the way to develop subunit vaccines to curb dengue infections (1). The recent findings in structural biology of dengue virus provide useful insight in determining drug targets to design anti-virals (2, 3). The plethora of information on various stages of dengue viral life cycle such as virus entry, membrane fusion, RNA genome replication, assembly and release, reveals the new approaches for the development of novel targets and stable drug delivery mechanisms (4-6). The enveloped virus enfolds ~11kb single stranded RNA genome of positive

sense with 5'cap which acts as both mRNA and template for the production of minus strand intermediate (5). The poly cistronic mRNA of DENV is flanked by the non-translated sequences namely 5'-NTR (100 nt) and 3'-NTR (450 nt) (7). In particular, six sequences in both 5' and 3'-NTRs are conserved among all the four DENV (8). Both the NTRs harborcis acting elements such as Cyclization sequences (CS), UARs (Upstream AUG region) and DARs (Downstream AUG region) facilitating the long range RNA-RNA interactions, which is necessary for the complete viral replication and translation (9, 10) as evidenced by the mutation studies within the CS in either region (11, 12). This is also evidenced by the inhibitory effect of artificial micro RNAs (amiRNAs) on DENV replication by targeting their 5'-CS (13).

Several efficient and high-throughput drug candidates such as mycophenolic acid and ribavirin (14), amantadine hydrochloride (15), R5-F2-R4 peptide-conjugated phosphorodiamidate morpholino oligomers (16), RNA silencing (17), DNA vaccine (18, 19), have been investigated for inhibition of dengue virus replication by both *in-vivo* and *in-vitro* studies. Among these, synthetic plasmid or vector based small dsRNA molecules have showed significant breakthrough in inhibiting the viral replication in the host to limited range, but chemical and in-situ mediated RNA have resulted in the side effects on host machinery due to lack of stability (20, 21). As there is a demand for the safe and specific delivery of small dsRNA molecules that potentially inhibit the viral replication in situ, recombinant adenovirus (rAd) and recombinant adeno-associated virus (rAAV) based gene delivery system is a suitable and recommended option (22). RNA interference is an evolutionary conserved sequence specific RNA degradation mechanism mediated by long dsRNA (23). Several methods expressing siRNA have evolved over time and demonstrated significant advancement in inhibiting dengue viral replication such as genetically modifying *A.aegypti* to express siRNA (24), targeting conserved 5'-CS of the DENV (25), targeting cellular surface receptors using specific siRNA (26) and AAV mediated siRNA delivery system (27).

In our earlier study a panel of 12 sh RNA (short hairpin RNA) constructs were studied to target the conserved sequences within the 5' and 3'-NTRs (non-translated regions) of DENV and screened them to determine the inhibition potential of the constructs. Among the 12 sh-5b RNA constructs, one construct named as sh-5b exhibited most promising inhibition and attenuated the replication of all four dengue serotypes with the average inhibition of 88% (28). Therefore, the present investigation is aimed to study the optimization (selection) and stability of sh-5b retention in recombinant adenovirus (up to 24 months) for siRNA delivery system to down-regulate the replication/translation of DENV-2. Briefly, the sh-5b RNA initially cloned into a vector pLKO.1 TRC to develop sh-5b RNA encoding plasmids. The expression cassette of the most effective sh-5b RNAwas then cloned into a replication defective human adenovirus type 5 vector (AdV5) named as pADEasy1 which is rendered replication defective by the deletion of E1 and E3 genes. Upon transfection of AD-293 cells with this construct, a recombinant adenovirus (rAd) harboring sh-5b RNA cassette to target the conserved site within the 5'-NTR of DENV-2 serotype was produced.

Materials and Methods

Cell lines and viruses: AD-293 (human AdV5 transformed human embryonic kidney 293 cells; HEK293), Monkey kidney Vero cell lines. DENV2 (New Guinea C, AF038403). *E. coli* strains DH5á, BJ5183 (Invitrogen, Carlsbad, CA), QIAamp Mini Pre- RNA kit & QIAmp viral RNA mini kit (Qiagen), TITAN one tube RT-PCR kit (Roche).

Construction of recombinant sh-5b adenovirus: The sh-5b RNA was cloned to downstream of the human U6 promoter in the vector pLKO.1 TRC between *Age*I and *Eco*RI restriction sites. The sh-5b RNA expression cassette was retrieved from the pLKO.1 sh-5b RNA construct and cloned into the pShuttle vector

to facilitate the homologous recombination with pAdEasy-1 plasmid. The sh-5b RNA expression cassette was inserted into the E1 region of pAdEasy-1 by *in vivo* homologous recombination in *E.coli* BJ5183. The recombinant Ad plasmid was linearized by using *Pac I* and transfected into the AD-293 cells to produce sh-5b RNA expressing rAd virus. Three different rAds, one with sh-5b cassette, second one with sh-N cassette and third without sh-insert were generated and verified by gene specific PCR, restriction enzyme analysis (28).

***Gene specific PCR at different intervals*:** To prove the consistent stable retention of sh-5b insert in recombinant adenovirus, the gene specific PCR was done at 12 and 24 months. At regular intervals of time viral DNA isolated by using HIRT lysis buffer and quantified the DNA by using Nano drop to make final concentration is ~500ng/ µl.

One-step RT-PCR: Vero cells ($3.5x10^3$ cells/well) seeded in one day in advance in 96-well plate were infected with rAdsh viruses. Each infected well received 50µl of 5% FCS + 1X DMEM after 2hrs of incubation at 37°C in 10% CO_2 and after 24h, infected with 50µl of DENV-2 (300pfu/well). After 2hrs 200µl of 5% FCS + 1X DMEM was added to each well and incubated. The cell culture supernatant was collected on 6th day post infection (d.p.i) and viral RNA was isolated using QIAamp Viral RNA kit (Qiagen) as per the manufacturer's protocol. The cDNA synthesis and PCR was performed in one-step by using D1 (5'-TCAATATGCTGAA ACGCGCGAGAA ACCG-3') and D2 (5'-TTGCACCAAC AGTCAATGTC TTCAGGTTC-3') primers using TITAN one-step RT-PCR kit.

Results

***Designing of potential sh-5b RNA target*:** The DENV-2 full-length genome (~11 kb) was analyzed using ClustalW, a web based program to identify stretches (21 nts) of highly conserved and overlapping sequences among the multiple DENV serotypes. Further, web based siRNA selection tool (siRNA, wizard) was explored to identify and design potential stable siRNAs to target multiple DENV serotypes. On comprehensive analysis of conserved regions, the regions in 5¹-NTR and 3¹-NTR are found to be potential targets for the present study. This has led us to identify the promising sh-5b RNAs and its subsequent cloning in specific vectors for the expression of sh-5b RNA sequences within the cells. The sh-5b RNAs were cloned downstream to the human U6 promoter in the vector pLKO.1 TRC between *Age*I and *Eco*RI restriction sites. The negative control with scrambled sh-5b RNA sequence (shN) insert was also constructed in the same manner.

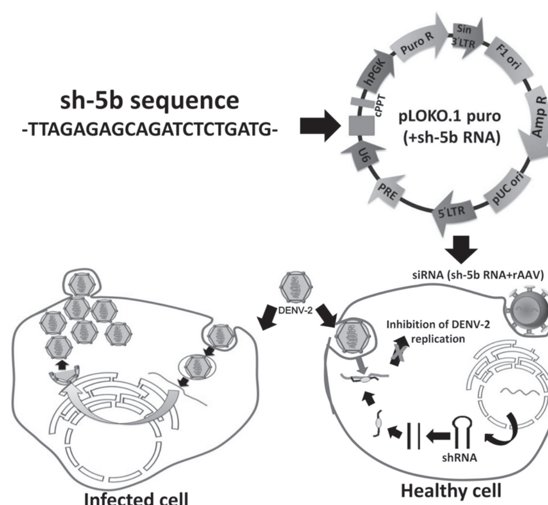

Fig. 1. Conserved sequence of sh-5b and construction of recombinant plasmid to target DENV-2 viral replication inhibition.

***Stability studies at different intervals*:** The insert specific PCRs were carried out after 24 and 48 months, to confirm the stability of sh5b and shN gene expression cassette into the adenoviral genome. The predicted band of ~1.2 kb using insert-specific primers with rAd-sh5b and rAd-shN genomic DNA, whereas no amplicon with the wild type (*wt*) AdV5 genome indicated the stable integration of 'sh' expression cassette.

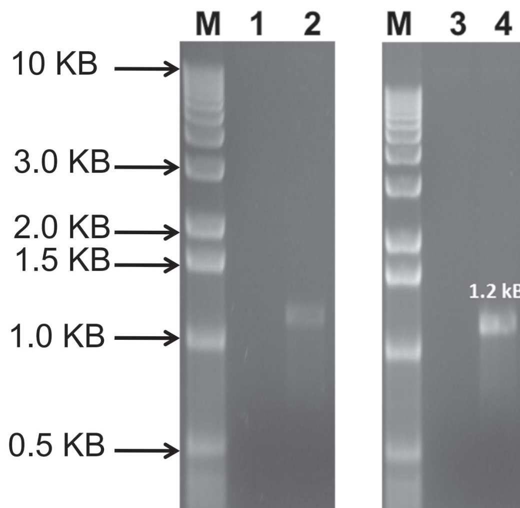

Fig. 2. Stability of sh-5b RNA verified at different time intervals (12 and 24 months) by gene specific PCR. Lane 1 and 3 are wild type adenovirus, lane 2 and 4 are sh-5b inserted adenovirus, PCR amplified at 12 and 24 months.

One step RT PCR optimization for DENV-2: One step RT-PCR was optimized with the total cellular RNA and culture supernatant RNA harvested from Vero cells (Mock cells) on Day 6 to use as a negative control. Positive control for the one-step RT-PCR done with the total cellular RNA and culture supernatant RNA harvested from Vero cells on Day 6, challenged with DENV-2. It is evident from the FIG. 3 in which the negative control is not showing any band, but the positive control is showing the clear band. One-step RT-PCR optimization done with triplicate wells using D1 and D2 primers.

One-step RT-PCR to evaluate the effect of rAd sh-5b on DENV-2 replication: DENV possess ~11 kb single stranded plus-sense RNA genome. Confirmation of sh-5b RNA on replication inhibition of DENV-2 by one step RT-PCR. RT-PCRs were performed with the total cellular RNA and culture supernatant RNA harvested from Vero cells (pre-infected with rAd-sh5b virus prior to DENV-2 challenge) on Day 6 post-DENV infection to determine the effect of rAd-sh5b on DENV-2 replication. One-step RT-PCR done with the culture supernatant using D1 and D2 primers. It is evident from the FIG. 4 that rAd-sh5b is effectively inhibiting the DENV-2 replication.

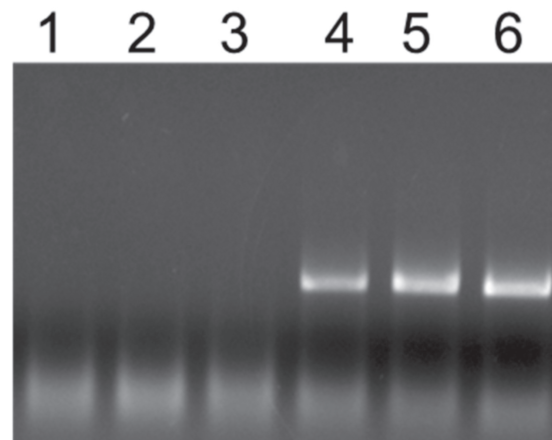

Fig. 3. One step RT PCR optimization for DENV-2. Lanes1,2,3 – Mock samples (only cells without dengue infection) 4,5,6 – Cells with dengue infected (Dengue 2)

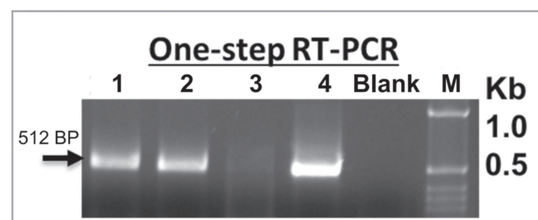

Fig. 4. A predicted band of 512 bp is observed in lane 1 (Virus control), lane 2 (rAd-shN) and lane 4 (positive control; DENV-2 RNA) whereas rAd-sh5b (lane 3) produced a diminished band of the corresponding size. 'M' corresponds to 1 kb DNA ladder.

Discussion

Though, the sh-5b RNA constructs effectively suppressed the DENV replication in transfected cells. However, the introduced siRNA loses its inhibitory effect and it gets degraded in a cellular environment over a period of time (21). The safe and consistent expression of the sh-

5b RNA constructs in the wide range of cells and its stable maintenance are necessary criterion. Adenoviral vectors have gained importance as drug-delivery systems for efficient gene silencing because of their potential to infect wide spectrum of cell type independent of active cell division and also because of their tendency to grow in high titers (29, 30). Further, it has been evidenced that the infection with a recombinant adenovirus vector, AdV5, containing sh-5b RNA does not significantly affect cellular mechanisms such as endogenous RNAi and interferon pathways (31). We created human U6 promoter driven rAd-sh-5b RNA system where in, the sh-5b expression cassette was cloned into the E1 region of rAd vector and this recombinant adenovirus was propagated in AD-293 cells. The schematic representation of rAd-sh-5b RNA delivery system in which E1 region is replaced by the sh5b expression system (rAd-sh5b), scrambled sh-RNA (rAd-shN) and empty rAd vector (rAd-E) is depicted in Fig. 1.

The adenoviral vector (maintained epichromosomally) used in this study is replication defective as it lacks E1 genes leading to extremely low levels of expression of virus-associated RNAs (VA-RNAs), which in turn has been shown to diminish their potential stability up to 24 months to interact with RISC and interfere with the RNAi pathway. Further the in-vivo studies in mice showed that adenoviral vectors can deliver adequate sh-5b RNAs to mediate inhibition of gene expression without overburdening the silencing machinery. This is consistent with our observation that the DENV replication is not affected by the rAd vector alone and thereby signifying the use of AdV5 as a suitable vector for mediating gene silencing via the delivery of exogenous effectors of RNAi into the Vero cells. Yet another important criteria which lagging behind is the absence of proper animal model to study the DENV pathogenesis. Therefore, new strategies are required for resurgence of the siRNA of either host origin or of introduced through vectors against these fatal viruses.

Conclusions

In the comprehensive analysis of sh-5b RNA targets in DENV-2 genome, we successfully repeated the potential sh-5b RNA target site within the 5¹-NTR region after 24 months stability study. The gene stability for 24 and 48 months of sh-5b RNA in inhibiting DENV-2 replication was examined by gene specific PCR. With this, we conclude that rational design of potential sh-5b RNAs targeting the conserved sequences and the development of efficient and safe system to deliver the exogenous effectors of RNAi will be useful in therapeutic intervention to combat DENV infections. The present investigation could be further extended to evaluate the effectiveness of the rAd-sh viruses in DENV challenged primary cultured cells and *in vivo* models.

Author Disclosure Statement : All the authors confirmed, there is no conflict of interest. No competing financial interests exist.

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Vol. 10 (1) 20-28 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Sorbitol and Sucrose- Induced Osmotic Stress on Growth of Wheat Callus and Plantlet Regeneration

V. K. Mishra[1*] and R. M. Singh[2]
[1]School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221005, India
[2]Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi-221005, India
*For Correspondece - mishravkbhu@gmail.com

Abstract

Scutellar somatic embryos are most commonly used as a target tissue for transformation and regeneration of transgenic wheat plants. Tissue culture responsiveness of elite varieties of wheat is one of the critical factors limiting high-frequency transformation. This study investigated the influence of sorbitol and sucrose-induced osmotic stress on growth and plantlet regeneration in callus cultures of wheat *(Triticum aestivum L.* em Thell). Immature embryos, at early-medium milking stages, of field grown wheat cvs., HUW206, HUW234, Sonalika and HD2009, were induced to form the callus on MS medium supplemented with 2, 4-D and 3% sucrose alone/or in a combination of sucrose and sorbitol. Fresh weight of callus was significantly promoted when 1.5% (w/v) sorbitol was added to the MS medium in conjunction with 3% (w/v) sucrose. However, higher concentration of sorbitol concentration (3% w/v) in conjunction with 3% (w/v) sucrose did not further improve fresh weight. Sorbitol in combination with sucrose caused a decrease in the water content from calli and promoted embryogenic callus formation. After 4 weeks, a high frequency (10-25%) shoot formation was obtained on MS media, IAA 1 mgL^{-1} and zeatin 1 mgL^{-1} in supplementation with sucrose and sorbitol. However, optimum plantlet regeneration (45-65%) was found on subsequent subculture on regeneration media containing 3% (w/v) sucrose and 3% (w/v) sorbitol. Osmotic stress induced by the combination of sucrose and sorbitol is beneficial for embryogenecity of the friable calli and high-frequency plantlet regeneration.

Key words : *In vitro* selection, NaCl tolerance, Wheat, *Triticum aestivum* L.

Introduction

Wheat is the major staple food crops of the world occupying 17% of the world's cultivatable land (over 200 million hectares) (1). Due to climate change and adverse environmental conditions, conventional breeding is facing a genetic bottleneck in achieving goals of sustainable agriculture. Also, there is evidence of yield plateaus or abrupt decreases in the rate of yield gain (2). In India, wheat production in the year 2014-15 estimated at 90.78 million tonnes which is lower by 5.07 MT as compared to 2013-14. Biotechnology can be integrated with the conventional breeding to build resilient cultivars to mitigate climate change and adverse environment.

The development of efficient and reproducible *in vitro* tissue culture and plant regeneration protocols is a prerequisite for successful application of genetic transformation and regeneration of transgenic wheat. Embryogenic calli derived from scutellum are the most commonly used target tissue for wheat genetic transformation with particle bombardment or gene transfer by Agrobacterium (3-5). Although various explants sources, such as anthers (6), immature inflorescences and immature embryos (7-9), mature embryos (10-

14), immature leaves (15), mesocotyls, and apical meristems (16) have been used for callus culture in wheat. The highest frequencies of callus induction and plantlet regeneration have been obtained from the culture of immature embryos of wheat (8, 9). Despite several attempts to replace immature embryos with other explants sources, regeneration capacity from alternative explants has been low (17, 18). Till date, immature zygotic embryos remained best explant sources for embryogenic callus and plantlet regeneration in wheat. Many factors, such as developmental stage of immature embryo, culture media, genotype physiological status of the donor plants (19), the culture medium, plant growth regulators and stresses play important during somatic embryogenesis (20).

High-frequency embryogenic callus formation and embryoids development were obtained by doubling the concentration of MS salts (8), the addition of different carbohydrate sources, such as sucrose, maltose, mannitol and sorbitol and osmotic stress (21-26). Osmotic stress affects plant cells growth and physiological metabolism. However, the mechanism of osmotic stress inducing shoot regeneration has not been well investigated in wheat. Keeping in view of suggested role of osmotic stress on somatic embryogenesis, it was envisaged to investigate the effect of high osmoticum on plant regeneration in wheat. In the present study, experiments were conducted on optimization of plantlet regeneration in wheat tissue culture using the combination of sucrose and sorbitol.

Materials and Methods

***Plant Materials*:** Four elite Indian varieties of wheat cv. HUW 206, HUW 234, Sonalika and HD 2009 were used for *in vitro* tissue culture experiments. Immature embryos of field grown plants were used as starting material for *in vitro* culture.

***Callus Induction*:** Green caryopses were removed from ear heads followed by surface sterilization using 70 % ethanol, and saturated solution of sodium hypochlorite (7% w/v) for 30 s. These were then washed with several changes of sterile distilled water. Following surface sterilization, translucent immature embryos, approximately 1.5 mm in length and 1.3 mm long, were aseptically excised from green caryopses under a stereo-microscope in a laminar flow at early-medium milking stages. Subsequently, they were transferred to sterilized MS media (Murashige and Skoog (1962) (27) supplemented with 3% (w/v) sucrose and 2, 4-D 2 mgL^{-1} and solidified with 0.8 % (w/v) for callus induction. Immature embryos were cultured in Petri-dishes (90 x 15 mm) with the scutellum up and the embryo axis kept in contact with induction medium. The cultures were kept in a cooled incubator at 25°C ± 2°C. After three weeks of culture, the frequency of callus induction (CI), embryogenic callus formation (EC) and precocious germination of immature (PGIE) and somatic embryos (PGSE) were recorded.

***Histological study*:** The somatic embryogenesis in wheat was studied by histological observations of embryogenic calli sampled at regular intervals. The specimens were fixed in a formalin-acetic acid-alcohol (FAA) solution (5% formol, 5% acetic acid, 90 % of 70% ethyl alcohol; *v*/*v*) for at least 24 h at 4°C. Then, they were dehydrated through a graded series of ethanol and finally embedded in paraffin wax. The sections of 10 µm were cut using a rotatory microtome. Following this, they were mounted on glass slides and stained for histological analysis following the protocol of Rao (1990) (28).

***Culture Maintenance*:** After three weeks of initial callus induction, compact and nodular callus-clumps were divided into 3-4 mm sized pieces and transferred to different MS supplemented media: (a) MS media supplemented with 3% sucrose; (b) MS media supplemented with 3% sucrose+1.5% sorbitol and 2,4-D 2 mgL-1, and (c) MS media supplemented with 3% sucrose+3% sorbitol. The Frequency of friable embryogenic callus formation was scored on the basis of friable embryogenic callus formed per total number of embryogenic callus –clumps placed in culture. After three weeks of culture,

data were recorded for fresh weight and dry weight, and the percentage water content of callus was determined as:

$$\text{Percent water content of callus} = \frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}}$$

Dry weight was determined after drying the sample to a constant weight at 80 °C.

***Plantlet regeneration*:** For regeneration, calli were transferred to MS medium with respective carbohydrate sources (sucrose 3%, sucrose 3%+sorbitol 1.5% and sucrose 3%,+3% sorbitol) supplemented with IAA 1 mgL^{-1} and zeatin 1 mgL^{-1} in a culture room maintained at 25^0C under 16/8h light/dark photoperiod. Illumination was provided by white fluorescent tubes at a photon density 70 µmol photons $m^{-2} s^{-1}$. After four weeks, plantlet regeneration frequency was calculated as the percentage of calli forming shoots. In another set of experiment, calli growing on respective carbohydrate sources were transferred to MS medium with 3% sucrose with sorbitol (1.5% and 3%, each separate treatment) supplemented with IAA 1 mgL^{-1} and zeatin 1 mgL^{-1}, and 0.8% agar in a culture room maintained in above conditions. The plantlets with well-formed roots were transferred to a sterile mixture of vermiculite containing soil (3:2) for hardening before transfer to soil.

Statistical Analysis: Data were analyzed in 2 x n Chi-square (x^2), and The individual comparisons were analyzed in 2×2 contingency table at 5% level of significance.

Results and Discussion

In the study, immature embryos (early-medium milking stages, size ~ 1.5 mm in length) readily formed the callus on MS medium supplemented with 2, 4-D 2 mgL^{-1}. Immature zygotic embryos, as well as somatic embryos have shown a tendency to germinate precociously during *in vitro* culture. Precocious germination is the development process of the somatic embryo before its complete maturation has taken place (29). In the preliminary experiment, it has been observed that precocious germination of embryos (zygotic and somatic) is influenced by genotypes. Precocious germination is an undesirable trait in tissue culture, because its expression limits the embryogenic potential of the culture. Once precocious germination is initiated, somatic embryos cannot multiply to give rise to secondary embryogenic tissues (30). After three weeks of culture, microscopic observation of the callus revealed the presence of a mixture of two types of callus: a compact, nodular embryogenic callus and a friable, watery and translucent callus (Fig. 1). In this work, all the wheat cultivars showed high-frequency callus induction (CI), ranging from 70 to 82 percent, which was independent of genotype in a Chi-square test ($p< 0.05$) (Table 1). However, the proportion of embryogenic callus formation (EC) was significantly different in wheat cultivars (d.f. 4, Chi-square test ($p< 0.05$) (Table 1). The significant influence of the genotype on embryogenic callus formation has also been observed in other studies (21, 31, 32).

Hormones play an important role in zygotic embryo and somatic embryogenesis. ABA is known for prevention of precocious germination. Approximately 90% of endogenous ABA in excised immature soyabean embryos diffused into ABA-free solution within 2 days (33). An abundant embryo protein, early methionine-labelled (Em) and wheat germ agglutinin (WGA) accumulate during mid to late stages of embryogenesis at a time when endogenous ABA levels are high in the kernel. These proteins disappear when the embryos are cultured in the absence of ABA and the same time embryos germinate prematurely (34). Therefore, it is concluded that precocious germination of cultured immature embryos may be related to loss of endogenous ABA through diffusion into ABA free medium. When immature embryos at 14 days post anthesis (DPA) were dissected from immature grains and cultured on nutrient medium, they commenced precocious germination immediately. This germination was

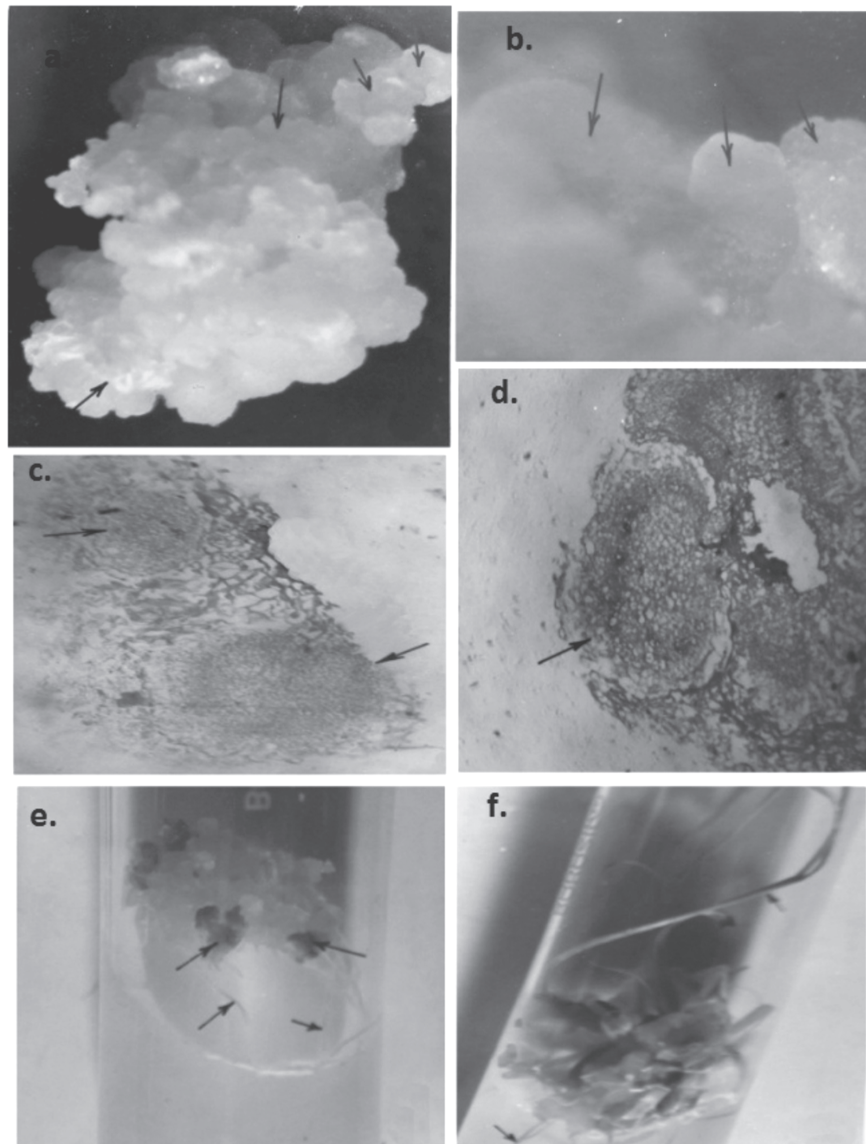

Fig. 1. Embryogenic callus of wheat at different developmental stages of embryoids and plantlet regeneration (a. embryogenic callus; b. A portion of calli showing somatic embryos at globular stage; c. Section of embryo at the globular stage; d. Post globular development; e. Embryogenic calli showing green spots (at early regeneration stage); and f. Plantlet regeneration.

prevented, not only by the inclusion of abscisic acid in the medium but also by the inclusion of osmotically active agents (35). There are reports indicating that osmoticum and/or exogenous ABA can prevent precocious germination, and enhance embryogenic callus formation (9,21,34,36-37).

Effect of Sugar and Sorbitol Induced Osmotic stress on Callus Growth: In most of the cereals, the subculture of primary embryogenic culture

Table 1. Frequency of precocious germination immature embryo (PGIE), callus induction (CI), embryogenic callus formation (EC) and precocious germination of somatic embryos on MS medium supplemented with 2, 4-D 2 mgL^{-1}

Genotypes	PGIE	CI	EC	PGSE
HUW 206	0.30 C	80 A	0.65 A	0.06 CD
HUW 234	0.56 B	75 A	0.50 B	0.12 BC
Kalyansona	0.06 D	78 A	0.70 A	0.04 D
HD 2009	0.84 A	70 A	0.25 C	0.50 A
Sonalika	0.20 C	82 A	0.75 A	0.20 B

Data were analyzed by chi -square test (χ^2) test in 2×n contingency table, and the pair- wise comparison each column were made in 2×2 contingency table. The values in the column followed by the same letter are not significantly different $p< 0.05$. PGIE=precocious germination of immature embryo; CI= callus induction; EC= yellow scutellar callus formation; PGSE= precocious germination of somatic embryos.

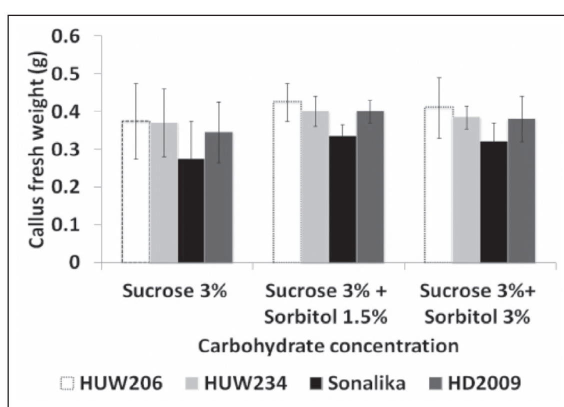

Fig. 2. Fresh weight of calli of four different cultivars of wheat on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

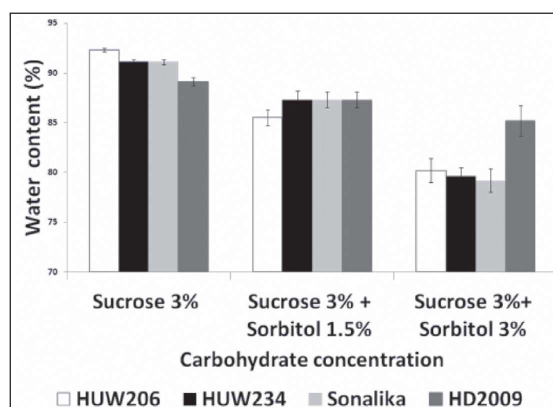

Fig. 3. Water content in calli of four different cultivars of wheat on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

leads to the formation of friable calli. A cluster of somatic embryos, obtained on callus induction medium, on subsequent subculture on combinations of sucrose and sorbitol formed friable callus. In the present work, fresh weight of friable callus was significantly promoted when 1.5% sorbitol was added to the MS medium in conjunction with 3% sucrose. With 1.5% sorbitol, the percent increase in fresh weight was highest in wheat cv. Sonalika (21.8%) followed by HD2009 (15.9%) and HUW206 (13.3%) and least in HUW234 (8.1%) as compared to fresh weight response to 3% sucrose. However, an additional increase in sorbitol to 3% in conjunction with sucrose (3%) did not further improve fresh weight. It appears that the promotory effect of sucrose on friable callus growth diminished with high concentrations of sorbitol, above 1.5% (Fig. 2). Generally, as the concentration of sorbitol increased, the water content of friable callus decreased. When 1.5% sorbitol was added in conjunction with 3% sucrose in the medium,

water content decreased ranging from 2 to 7.3 %, highest in wheat cv.HUW 206 followed by HUW 234 (4.7%), Sonalika (4.1%), and least in HD2009 as compared to water content at 3% sucrose. The Water content of wheat friable callus significantly decreased with further increase in sorbitol to 3%, ranging from 4.3% to 13.1%, highest in wheat cvs. HUW206 and Sonalika followed by HUW 234 (12.6%) and least in HD2009 (Fig.3).

Improvement in embryogenic callus formation in wheat has been demonstrated by application of various sugars, such as sucrose (38) and maltose (39), sorbitol (40), mannitol (41), and PEG (41) and as well as high MS salt concentration (8, 9). Sorbitol used in conjunction with sucrose had beneficial effects on morphogenesis of rice (42,43), wheat (40, 44), and barley cultures (40). However, there is no evidence that sorbitol is utilized as a carbon source in these species. Rather, sorbitol was to metabolized in maize and imparted beneficial effects on embryogenic callus formation(45). It is reported that water-stress induced plantlet regeneration in rice (46-48).

***Effect of Sugar and Sorbitol Induced Osmotic Stress on Plantlet Regeneration*:** Friable- calli of wheat growing on sucose-sorbitol containing media showed plantlet regeneration ranging from ranged from 17 to 25% on sugar and sorbitol supplemented MS media after four weeks of culture (Fig. 4). However, high- frequency plantlet regeneration occurred on prolonging osmotic stress of calli (eight weeks of culture) under sucrose-sorbitol induced osmotic stress (Fig.5).

Under osmotic stress, the incessant supply of endogenous ABA favoured the development and maturation somatic embryos leading to high-frequency somatic-embryo to plant conversion. It is reported that osmotic stress for an extended period to the cultured embryos of wheat has promoted expression of Em genes under continued presence of embryonic ABA (35). Another report also supported embryonic ABA synthesis in wheat under drought condition (49).

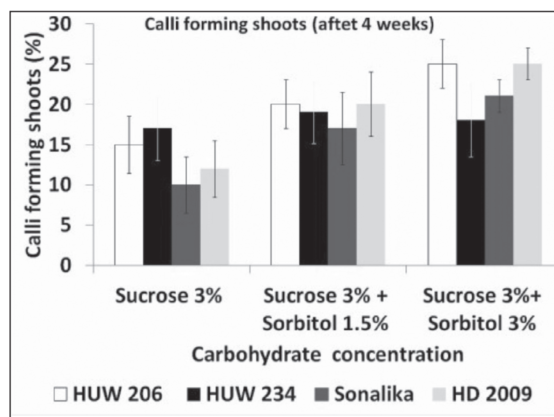

Fig. 4. Calli forming shoots after four weeks of culture on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

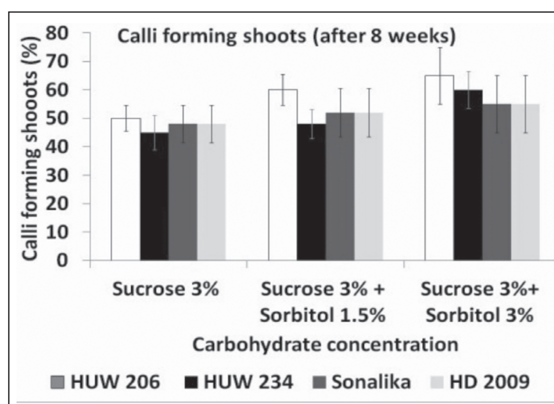

Fig. 5. Calli forming shoots after eight weeks of culture on MS medium supplemented with 3% sucrose alone, and in conjunction with sorbitol supplementation (1.5% and 3%)

Thus, sucrose and sorbitol-induced osmotic stress enhanced somatic embryogenesis and shoot regeneration in immature embryo culture of wheat.

Conclusion

ABA-mediated stimulation of somatic embryogenesis is implicated in wheat. However, the mechanism of osmotic stress induced signaling on regeneration in wheat still needs to be further explored.

Acknowledgements

The part of the work has been supported under Assistance programme (SAP) sponsored by University Grants Commission (U.G.C.), N. Delhi (India).

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Vol. 10 (1) 29-36 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Phytoconstituents from the Leaves of *Adenanthera Pavonina* Linn And *Erythrina variegata* L. (*Syn.*= *Erythrina indica* Lam)

Md. Mujahid, Md. Azizur Rahman*, Hefazat H. Siddiqui, Arshad Hussain, Vaseem Ahamad Ansari and Mohammad Irfan Khan
Bioactive Research Laboratory, Department of Pharmacy, Integral University, Lucknow, Uttar Pradesh 226026 (India).
*For correspondence - aziz0pharmacy@gmail.com

Abstract

Adenanthera pavonina Linn and *Erythrina variegata* Lam are being used in the Indian traditional systems of medicine for the cure of variety of ailments. Present study was designed to isolate and characterize phytoconstituents from the leaves of *A. pavonina* Linn and *E. variegata* Lam. Methanolic extracts of the leaves of *A. pavonina* and *E. variegata* were prepared and slurry on silica gel was prepared from these extracts separately. Silica gel was packed in chromatography column and dried slurry was loaded. The column was eluted successively with different solvents in the order of increasing polarity. Chromatographically identical fractions were combined together and concentrated. Isolated phytoconstituents were crystallized and characterized by UV, IR, ^{1}H-NMR, ^{13}C-NMR and MS spectroscopy. Isolated phytoconstituents 1, 2, 3 and 4 were characterized as *n*-tricosanol, α-D-glucopyranosyl-(2→1')-α-D-glucopyranosyl-(6'→2' 2)-α-D-glucopyranosyl-(6"→1' 2 →2)-α-D-glucopyranoside, hexatetracontan-1-ol and *n*-octanyl-1β-D-glucopyranosyl-(6"→ 1')-β-D-glucopyranoside respectively. From the present study, it can be concluded that the existing knowledge concerning the phytoconstituents from the leaves of *A. pavonina* and *E. variegata* may be enlarged by the current phytoconstituents investigation which is valuable as these drugs are being used in the Indian traditional systems of medicine.

Keywords: *Adenanthera pavonina, Erythrina variegata,* isolation, phytoconstituent, column chromatography

Introduction

Adenanthera pavonina Linn belonging to the family Fabaceae is normally known as red wood (1). Traditionally, its ground seed is used for the treatment of diverse ailments such as inflammation, arthritis, rheumatism, convulsion, epilepsy, paralysis, spasm, cholera, boils, blood disorders, hepatotoxicity and indigestion (2, 3). Scientific investigation on *A. pavonina* showed that the plant extracts have anti-inflammatory, analgesic, antifungal, anti-oxidant, antihyperlipidemic, anti-diabetic, cytotoxic and blood pressure lowering effects (4, 5, 6, 7). Its seed contains O-acetylethanolamine; leaves stigmasterol, dulcitol, octacosanol, glucosides of â-sitosterol; bark stigmasterol glucoside; and pods steroids, saponins and glycosides (8, 9, 10). Pavonin, a phytoconstituent with lactone ring and exo-cyclic double bond was also isolated from its methanol soluble part (11).

The plant *Erythrina variegata* Lam belonging to the family Papilionaceae is normally known as Indian coral tree in English and Mandara in Hindi. Traditionally, the leaves are used as diuretic, laxative, galactagogue, emmenagogue, antihelmintic and in the treatment of joint pain (12, 13, 14). Scientific investigation on *E. variegata* showed that the

plant extracts have central nervous system effect, anti-osteoporotic, cardiovascular, cytotoxic, antiulcer, anthelmintic, diuretic, analgesic and antioxidant activities (15, 16, 17, 18). The plant contains sterols like campesterol, β-amyrin, β-sitosterol; alkaloids like N-norprotosinomenine, erysodienone, protosinomenine, 3-erythroidine, erythraline, erysopine, erythramine, erysotrine, erysodine, erythratine, hyparphorine, N,N-dimethyltryptophan; isoflavones like indicanines D and E; flavonoids like genkwanin, apigenin, iso-vitexin, saponarin, swertisin, 5-O-glucosylisoswertisin, 5-Oglucosylswertisin; and a triterpene betulin (19, 20, 21).

Present study was carried out to explore the plants *A. pavonina* and *E. variegata* with regard to their phytoconstituents and also to enlarge the existing knowledge concerning their phytoconstituents. Hence, present study describes the isolation and characterization of phytoconstituents from the leaves of *A. pavonina* and *E. variegata*.

Materials and Methods

Collection and authentication of plant specimens: Fresh leaves of the plants *Adenanthera pavonina* Linn and *Erythrina variegata* Lam were collected from Pallavaram, Chennai, Tamilnadu and authenticated by Plant Anatomy Research Center, National Institute of Herbal Science, Chennai (ref. no.: PARC/2011/954 and PARC/2011/955).

***Chemicals and instruments*:** All the solvents and chemicals used in the study were of analytical grade. Petroleum ether 60-80°C, methanol, formic acid, toluene, and diethyl ether were obtained from SD Fine Chem Pvt Ltd, Mumbai. Ethanol, Chloroform, Silica gel 60-120 mesh size, and Silica gels G were obtained from Merck Ltd, Mumbai. Melting point apparatus (Remi Equipments, India), Electronic balance (Sartorious, India), UV Spectrophotometer (160A UV-Vis, Shimadzu, Japan), FTIR Spectro-photometer (Bio-Red).

***Preparation of extracts*:** Collected leaves of the plants *A. pavonina* Linn and *E. variegata* Lam were washed with distilled water, dried under shade at room temperature and powdered to a coarse powder (500 g). The powder was packed in muslin cloth and subjected to Soxhlet extraction with methanol for 72 h at 50°C. Methanolic extracts were filtered through Whatmann No.1 filter paper and filtrates were concentrated to dryness under reduced pressure and temperature in Rotavapor. Extracts were stored in freezer and used for phytochemical isolation. Procedure was followed separately for both the plants (22).

***Preparation of slurry for column chromatography of the extracts*:** *A. pavonina* (10 g) and *E. variegata* (10 g) extracts were used for the preparation of slurry. The concentrated extract of the plant material was taken in a China dish and heated continuously on a water bath by gradual addition of methanol in small portions with constant stirring till desired consistency was obtained. A weighed quantity of silica gel for column chromatography was then added slowly with continuous mixing with a steel spatula until the whole methanolic solution of the extract adsorbed on silica gel particles. It was dried in air and the larger lumps were broken by rubbing between hands and finally passed through a sieve No 8 to get uniform particle size.

***Packing of column and isolation of phytoconstituents*:** A column of 3 ft height and 16 mm internal diameter was taken, cleaned properly and dried. The lower end of the column was plugged with non-absorbent cotton wool. The column was clamped and fitted in vertical position on a stand. The column was then half filled with petroleum ether 60-80°C. Silica gel for column (60-120 mesh size) was then poured in small portions and allowed to settle down and the dried slurry was loaded over the column and then eluted successively with different solvents in the order of increasing polarity. The developments and elution of the column were carried out with successive series of different solvents in various combinations, such as petroleum ether (100%), petroleum ether:chloroform (75:25, 50:50, 25:75), chloroform (100%), chloroform:methanol (99:1,

98:2, 97:3, 95:5, 90:10, 80:20) and methanol (100%) to isolate the phytoconstituents.

***Homogeneity of the fractions*:** The fractions collected were subjected to thin layer chromatography (TLC) to check homogeneity of various fractions. Chromatographically identical fractions (having same R_f values) were combined together and concentrated. Isolated phytoconstituents were crystallized with suitable solvent system.

***Spectral characterization of isolated phytoconstituents*:** Ultraviolet (UV) spectra of isolated phytoconstituent were recorded on UV spectrophotometer in methanol at Faculty of Pharmacy, Integral University, Lucknow. Infrared (IR) spectra were recorded on FTIR spectrophotometer using KBr pellets. ^{1}H-NMR spectra were screened on Bruker spectrospin 400 MHz instrument using deutarated chloroform ($CDCl_3$) as solvent and tetramethylsilane (TMS) as internal standard. ^{13}C-NMR spectra were recorded on Bruker Spectrospin 400 MHz in 5 mm spinning tubes at 27°C. Mass spectra (MS) were scanned by effecting FAB ionization at 70 eV on a JEOL-JMS-DX 303 instruments equipped with direct inlet probe system. IR, ^{1}H-NMR, ^{13}C-NMR and MS spectral characterization were carried out at SAIF, Central Drug Research Institute (CDRI), Lucknow. TLC spots were visualized by exposure to iodine vapors and UV radiation.

Results

Structures of the phytoconstituents 1, 2 isolated from methanolic extract of the leaves of *A. pavonina* and phytoconstituents 3, 4 isolated from methanolic extract of the leaves of *E. variegata* are shown in figure 1.

***Spectral characterization of isolated phytoconstituents from Adenanthera pavonina*:** Elution of the column with chloroform:methanol (9:1) yielded yellowish mass of phytoconstituent (1) (110 mg). R_f value [Solvent system: chloroform:methanol (9:1)]: 0.46; mp: 120-125°C; UV λ_{max} (MeOH): 214 nm; IR v_{max} (KBr): 3401, 2925, 2841, 1647, 1216, 1096, 770 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 3.37 (2H, t, *J*= 6.0 Hz, H_2-1), 2.30, (2H, m, H_2-2), 2.02 (2H, m, CH_2), 1.72 (2H, m, CH_2), 1.58 (4H, m, 2×CH_2), 1.41 (4H, brs, 2×CH_2), 1.29 (6H, brs, 3×CH_2), 1.25 (2H, brs, 11×CH_2), 0.87 (3H, t, *J*=6.9 Hz, Me-23); ^{13}C-NMR ($CDCl_3$): δ 62.95 (C-1), 32.94 (CH_2), 31.84 (CH_2), 29.90 (16×CH_2), 29.57 (CH_2), 29.37 (CH_2), 22.90 (CH_2), 14.32 (C-12), 75.08 (C-22), 69.84 (C-32), 73. 97 (C-42), 64.51 (C-52), 13.18 (C-12 2), 22.03 (Me-23); ESI-MS (*m/z*, rel. int.): 340 $[M]^+$ ($C_{23}H_{48}O$) (48.2).

Elution of the column with chloroform: methanol (9:1) furnished colourless crystals of phytoconstituent (**2**) (105 mg). R_f value [Solvent system: chloroform:methanol (9:1)]: 0.49; mp: 130-132°C; UV λ_{max} (MeOH): 264 nm; IR v_{max} (KBr): 3401, 3345, 3215, 3019, 2951, 2842, 1647, 1384, 1215, 1095, 759 cm^{-1}; ^{1}H-NMR (MeOD): δ 5.12 (1H, d, *J*=3.9 Hz, H-1α), 4.49 (1H, d, *J*=6.1 Hz, H-12), 4.06 (1H, d, *J*=5.1 Hz, H-12 2), 4.01 (1H, d, *J*=3.1 Hz, H-12 2 2), 3.95 (1H, m, H-2), 3.89 (1H, m, H-22), 3.84 (1H, m, H-5), 3.82 (1H, m, H-52), 3.80 (1H, m, H-52 2), 3.78 (1H, m, H-52 2 2), 3.76 (1H, m, H-22 2), 3.75 (1H, m, H-22 2 2), 3.72 (1H, m, H-3), 3.69 (1H, m, H-32), 3.65 (1H, m, H-32 2), 3.61 (1H, m, H-32 2 2), 3.59 (2H, m, H-4, H-42), 3.56 (1H, m, H-42 2), 3.54 (1H, m, H-42 2 2), 3.31 (2H, brs, H2-62 2), 3.25 (2H, d, *J*= 9.8 Hz, H_2-6), 3.13 (2H, d, *J*=5.1 Hz, H_2-62), 3.11 (2H, d, *J*=4.8 Hz, H2-62 2 2); ^{13}C-NMR ($CDCl_3$): δ 103.27 (C-1), 84.98 (C-2), 73.08 (C-3), 71.83 (C-4), 78.19 (C-5), 60.89 (C-6), 99.32 (C-12), 83.38 (C-22), 72.66 (C-32), 71.33 (C-42), 78.10 (C-52), 62.86 (C-62), 98.29 (C-12 2), 76.40 (C-22 2), 72.10 (C-32 2), 69.53 (C-42 2), 77.69 (C-52 2), 64.65 (C-62 2), 94.05 (C-12 2 2), 76.40 (C-22 2 2), 71.94 (C-32 2 2), 65.99 (C-42 2 2), 76.90 (C-52 2 2), 62.95 (C-62 2 2), ESI MS (*m/z*, rel. int.): 666 $[M]^+$ ($C_{24}H_{42}O_{21}$) (21.3), 504 (12.6) 342 (77.8), 324 (10.5), 179 (6.6).

***Spectral characterization of isolated phytoconstituents from Erythrina variegata*:** Elution of the column with chloroform:methanol (4:1) furnished colorless crystals of phytoconstituent (3) (130 mg). R_f value [Solvent

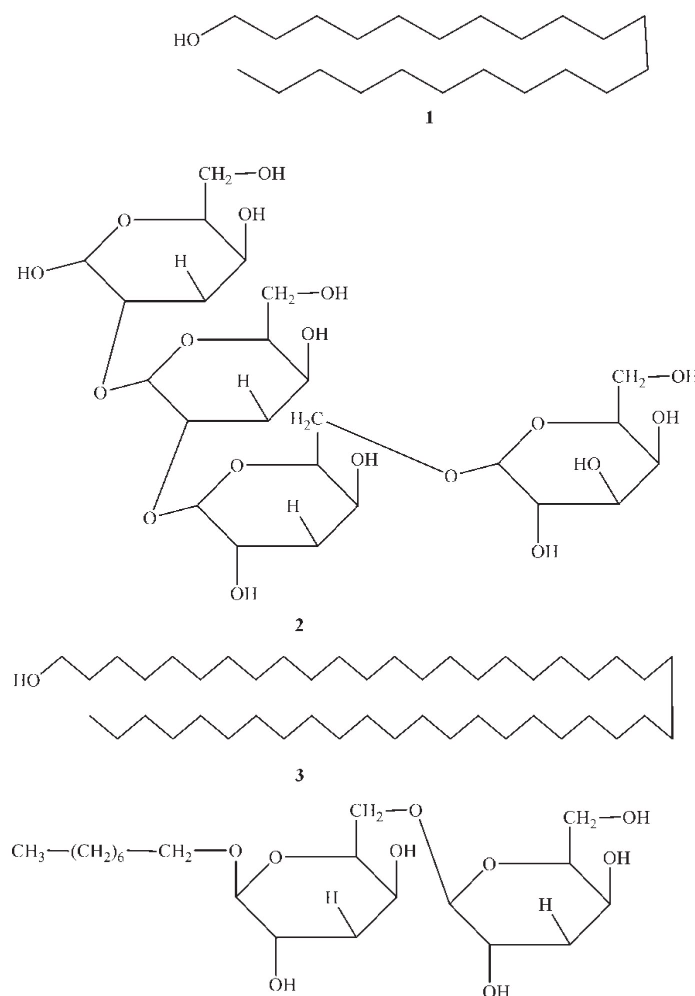

Fig. 1. Structure of isolated phytoconstituents tricosan-1-ol (**1**), α-D-glucopyranosyl-(2→1)-α-D-glucopyranosyl-(2'→2')-α-D-glucopyranosyl- (6''''→ 1'')-α-D-glucopyranoside (2), hexatetra-contan-1-ol (3), and *n*-octanyl-1β-D-glucopyranosyl-(6''''→ 1'''')-β-D-glucopyranoside (4).

system: chloroform: methanol (4:1)]: 0.41; mp: 125-130°C; UV λ_{max} (MeOD): 207 nm; IR υ_{max} (KBr): 3411, 2975, 2842, 2842, 1602, 1475, 1215, 1046, 928, 876, 758 cm^{-1}. ^{1}H-NMR (MeOD): ä 3.82 (2H, t, *J*=9.0 Hz, H_2-1), 2.05 (2H, m, H_2-2), 1.59 (2H, m, CH_2), 1.46 (2H, m, CH_2), 1.38 (6H, brs, 3×CH_2), 1.29 (74H, brs, 37×CH_2), 0.89 (3H, t, *J*=6.5 Hz, Me-46); ^{13}C-NMR ($CDCl_3$): ä 103.27 (C-1), 84.98 (C-2), 73.08 (C-3), 71.83 (C-4), 78.19 (C-5), 60.89 (C-6), 99.32 (C-12), 83.38 (C-22), 72.66 (C-32), 71.33 (C-42), 78.10 (C-52), 62.86 (C-62), 98.29 (C-12 2), 76.40 (C-22 2), 72.10 (C-32 2), 69.53 (C-42 2), 77.69 (C-52 2), 64.65 (C-62 2), 94.05 (C-12 2 2), 76.40 (C-22 2 2), 71.94 (C-32 2 2), 65.99 (C-42 2 2), 76.90 (C-52 2 2), 62.95 (C-62 2 2); ESI-MS (*m/z*, rel. int.): 662 $[M]^+$ ($C_{46}H_{94}O$) (100).

Elution of the column with chloroform: methanol (4:1) furnished colorless crystals of phytoconstituent (4) (110 mg). R_f value [solvent system: chloroform:methanol (4:1)]: 0.54; mp: 110-115°C; UV $\ddot{e}_{max}$ (MeOD): 244 nm; IR v_{max}

(KBr): 3401, 3343, 3019, 1645, 1425, 1384, 1216, 1095, 770 cm^{-1}; ^{1}H-NMR (MeOD): ä 5.17 (1H, d, *J*=7.8 Hz, H-12), 5.07 (1H, d, *J*=7.8 Hz, H-12 2), 4.34 (1H, m, H-52), 4.04 (1H, m, H-52 2), 3.89 (1H, m, H-22), 3.82 (1H, m, H-22 2), 3.73 (1H, m, H-32), 3.68 (1H, m, H-32 2), 3.55 (1H, m, H-42), 3.48 (1H, m, H-42 2), 3.34 (2H, t, *J*=11.4 Hz, H_2-1), 3.28 (2H, d, *J*=7.5 Hz, H_2-62), 3.22 (2H, d, *J*=9.3 Hz, H_2-62 2), 2.30 (2H, m, H_2-2), 1.61 (2H, m, CH_2), 1.28 (8H, brs, 4×CH_2), 0.89 (3H, t, *J*=6.6 Hz, Me-8); ^{13}C-NMR ($CDCl_3$): *ä* 66.05 (C-1), 30.93 (C-2), 30.08 (C-3), 29.91 (C-4), 29.19 (C-5), 25.83 (C-6), 22.69 (C-7), 14.16 (C-8), 99.32 (C-12), 73.58 (C-22), 72.15 (C-32), 71.37 (C-42), 74.43 (C-52), 64.68 (C-62), 85.03 (C-12 2), 72.69 (C-22 2), 71.98 (C-32 2), 69.57 (C-42 2), 73.88 (C-52 2), 60.92 (C-62 2); ESI-MS (*m/z*, rel. int.): 454 $[M]^+$ ($C_{20}H_{38}O_{11}$) (6.1) 180 (6.3).

Discussion

***Structural elucidation of isolated phytoconstituents from Adenanthera pavonina*:** Phytoconstituent (1) namely *n*-tricosanol was obtained as a yellowish mass from chloroform:methanol (9:1) eluent. Its IR spectrum showed absorption bands for hydroxyl groups (3401 cm^{-1}), unsaturation (1647 cm^{-1}) and long aliphatic chain (770 cm^{-1}). On the basis of mass and ^{13}C-NMR spectra, the molecular ion peak of 1 was determined at *m/z* 340 corresponding to the molecular formula of $C_{23}H_{48}O$. The ^{1}H-NMR spectrum of 1 showed a two proton triplets at δ 3.37 (*J*=6.0 Hz) assigned to anomeric H_2-1 proton. It also showed the three two proton multiplet at δ 2.30, 2.02 and 1.72 assigned to H_2-2 and one four proton multiplet at δ 1.58 protons. The other sugar protons appeared between δ 1.41 and 1.25. A three-proton triplet at δ 0.87 (*J*=6.0 Hz) was accounted to the methyl protons. The ^{13}C-NMR spectrum displayed signals for anomeric carbon at δ 62.95 (C-1), other carbons between δ 31.94 and 22.37. On the basis of these spectral data analysis, structure of 1 was characterized as *n*-tricosanol which is a new octacosanol like compound from *A. pavonina* but known in other plants (23, 24).

Phytoconstituent (**2**) namely α-D-glucopyranosyl-(2'!12)-α-D-glucopyranosyl-(2'→1")-α-D-glucopyranosyl-(6"→1"')-α-D-glucopyranoside was obtained as a colorless crystalline mass from chloroform:methanol (9:1) eluent. It gave positive test for reducing sugar and had IR absorption bands for hydroxyl group (3401, 3345 and 3215 cm^{-1}) with ester group (1647 cm^{-1}), pyranosyl (1384 cm^{-1}) and glycosidic linkage (1114 cm^{-1}). Vibration of a C¯O linkage has been shown to produce an absorption band at 1384 cm^{-1} supporting the presence of pyranosyl group in the phytoconstituent. On the basis of mass and ^{13}C-NMR spectra, the molecular ion peak of phytoconstituent 2 was determined at *m/z* 666 corresponding to the molecular formula of α-D-tetraglucoside ($C_{24}H_{42}O_{21}$). The ^{1}H-NMR spectrum of phytoconstituent 2 displayed a numeric proton signals on one-proton doublets at δ 5.12 (*J*=3.9 Hz), 4.49 (*J*=6.1 Hz), 4.06 (*J*=5.1 Hz) and 4.01 (*J*=3.1 Hz) as three two-proton double doublet at δ 3.25 (*J*=9.8 Hz), 3.13 (*J*=5.1 Hz) and 3.11 (*J*=4.8 Hz). The numeric single proton multiplets appeared from δ 3.95 to 3.54. The ^{13}C-NMR spectrum of phytoconstituent 2 exhibited signals for four anomeric sugar carbons between δ 103.27 to 84.98 and other sugar carbons in the range of δ 73.08 to 62.95. On the basis of these spectral data analysis, structure of phytoconstituent 2 was characterized as α-D-glucopyranosyl-(2'!12)-α-D-glucopyranosyl-(2→1")-α-D-glucopyranosyl-(6"→1"")-α-D-glucopyranoside. It is a new glucosidic phytoconstituent from *A. pavonina*. The results of the study on *Adenanthera pavonina* clearly indicates that its leaves contain *n*-tricosanol and α-D-glucopyranosyl-(2→1')-α-D-glucopyranosyl-(2"→1"")-α-D-glucopyranosyl-(6""→1"")-α-D-glucopyranoside which is supported by previous reports that the leaves of *A. pavonina* contain stigmasterol, dulcitol, octacosanol, glucosides of β-sitosterol (8, 9, 10). But, these isolated phytoconstituents; tricosanol and glucoside from *A. pavonina* are different from the alcohols and glucosides already reported in it.

Structural elucidation of isolated phytoconstituents from Erythrina variegata: Phytoconstituent (3) namely hexatetracontan-1-ol was obtained as a yellowish mass from chloroform:methanol (4:1) eluent. Its IR spectrum showed absorption bands for hydroxyl groups (3411 cm^{-1}), unsaturation (1602 cm^{-1}) and long aliphatic chain (758 cm^{-1}). On the basis of mass and ^{13}C-NMR spectra, the molecular ion peak of 3 was determined at *m/z* 662 corresponding to the molecular formula of $C_{46}H_{94}O$. The ^{1}H-NMR spectrum of 3 showed a two proton triplets at δ 3.82 (*J*=6.0 Hz) assigned to anomeric H_2-1 proton. It also showed the three two proton multiplet at δ 2.05 assigned to H_2-2 and 1.59 and 1.46. The other sugar protons appeared between δ 1.38 and 1.29. A three-proton triplet at δ 0.89 (*J*=6.5 Hz) was accounted to the methyl protons. The ^{13}C-NMR spectrum displayed signals for anomeric carbon at δ 62.95 (C-1), other carbons between δ 31.94 and 22.37. On the basis of these spectral data analysis, structure of phytoconstituent 3 was characterized as hexatetracontan-1-ol which is a new compound from *E. variegata* but known in other plants (25).

Phytoconstituent (4) namely *n*-octanyl-1β-D-glucopyranosyl-(6'→1")-β-D-glucopyranoside was obtained as a colorless crystalline mass from chloroform:methanol (4:1) eluent. It gave positive test for reducing sugar and had IR absorption bands for hydroxyl group (3401, 3343 and 3019 cm^{-1}) with ester group (1645 cm^{-1}), pyranosyl (1384 cm^{-1}) and glycosidic linkage (1216 cm^{-1}). Vibration of a C¯O linkage has been shown to produce an absorption band at 1384 cm^{-1} supporting the presence of pyranosyl group in the phytoconstituent. On the basis of mass and ^{13}C-NMR spectra, the molecular ion peak of phytoconstituent **4** was determined at *m/z* 454 corresponding to the molecular formula of β-D-glycoside ($C_{20}H_{38}O_{11}$). The ^{1}H-NMR spectrum of phytoconstituent **4** displayed a numeric proton signals on four one-proton doublets at δ 5.17 (*J*=7.8 Hz), 5.07 (*J*=7.8 Hz), 3.28 (*J*=7.5 Hz) and 3.22 (*J*=9.3 Hz), assigned to H-12 , H-12 2 , H_2-62 and H_2-62 2 . The two proton triplet at 3.34 (*J*=11.4) and a three triplet-proton at δ 0.89 (*J*=6.6 Hz) assigned to methyl proton. The numeric single proton multiplets appeared from δ 4.34 to 3.48. The ^{13}C-NMR spectrum of phytoconstituent 4 exhibited signals for four anomeric sugar carbons between δ 66.05 and 14.16, and other sugar carbons in the range of δ 99.32 to 60.92. On the basis of these spectral data analysis, structure of phytoconstituent 4 was characterized as *n*-octanyl-1β-D-glucopyranosyl-(6' →1")-β-D-glucopyranoside. It is a new glycosidic phytoconstituent. Previous reports on *E. variegata* clearly indicates that the plant contains sterols like campesterol, β-amyrin, β-sitosterol; alkaloids like N-norprotosinomenine, erysodienone, protosinomenine, 3-erythroidine, erythraline, erysopine, erythramine, erysotrine, erysodine, erythratine, hyparphorine, N,N-dimethyltryptophan; isoflavones like indicanines D and E; flavonoids like genkwanin, apigenin, iso-vitexin, saponarin, swertisin, 5-O-glucosylisoswertisin, 5-Oglucosylswertisin; and a triterpene betulin (19, 20, 21). The results of the study on *E. variegata* clearly indicates that its leaves contain hexatetracontan-1-ol and *n*-octanyl-1β-D-glucopyranosyl-(6'→1")-β-D-glucopyranoside suggesting the presence of two new phytoconstituents in *E. variegata*.

Conclusion

The present work characterized four phytoconstituents isolated from the leaves of *A. pavonina* and *E. variegata*. The existing knowledge concerning their phytoconstituents may be enlarged by the current phytoconstituents investigation which is valuable as these drugs are being used in the Indian traditional systems of medicine.

Acknowledgement

The authors acknowledge the help rendered by Head, CIF, Central Drug Research Institute (CDRI) Lucknow for scanning spectra and also acknowledge the help rendered by Prof (Dr.) Mohammed Ali, Faculty of Pharmacy, Jamia Hamdard, New Delhi for the interpretation of isolated phytoconstituents.

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Vol. 10 (1) 37-45 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Effect of Growth Regulator in MS Media on *In Vitro* Multiplication of Strawberry (*Fragaria* × *ananassa* Duch.) using Runner Tip Explants

Sharma Vikas Kumar *, Godara Anil Kumar and Goyal R. K.
Department of Horticulture, CCS Haryana Agricultural University, Hisar- 125004, Haryana
For correspondence - vksharma087@gmail.com

Abstract

Effect of different growth regulators on *in vitro* multiplication of strawberry (*Fragaria* × *ananassa* Duch.) cv. Camarosa through runner tip was studied with various combinations and concentrations IAA, BAP & Kinetin and IBA & NAA hormones using Murashige and Skoog media (MS). MS media with 0.5 µM IAA + 2.5 µM kinetin took minimum days (11.67) for shoot proliferation and produced maximum number of shoots/ culture (12.67) followed by 0.1 µM IAA + 2.0 µM kinetin. Application of IBA (2 mg/l) in MS media initiated maximum roots in minimal time (7.33). Maximum mean number of roots/ explant (9.33) and length of roots (6.17 cm) was also recorded in MS + IBA (2 mg/ l) followed by MS + IBA (1 mg/ l) media. The rooted plants transferred in cocopeat + perlite + vermicompost (3:1:1) showed maximum transplanting success (94.33%) while minimum from S_6 (63.00%). The outcome of the study is useful for *in vitro* multiplication and genetic engineering work in strawberry in future.

Key words: Growth regulators, *In vitro*, Micro-propagation, Runner tip, Strawberry

Introduction

The modern cultivated strawberry (*Fragaria* × *ananassa* Duch.) is one of the widely distributed soft fruit crop in the world due to its genotypic diversity, highly heterozygous nature and broad range of environmental adaptations (1). The mature fruits are quite delicious, refreshing, and attractive with distinct and pleasant aroma and healthy composition.In India, the cultivated area under strawberry is nearly 15600 ha (2).

Availability of elite planting material has been a barrier in expansion strawberry cultivation. Conventionally, strawberry is propagated by runners (3, 4), which is very labour intensive, time consuming and results in the transmission of over 30 viruses and phytoplasmic diseases (5, 6) and caused yield reduction may be up to 80% (7). The mass multiplication *in vitro* through tissue culture results in high yield of disease free plant material (8, 9) and proved to be the best alternative approach to conventional propagation method (10). The use of meristem tip culture for virus elimination is employed for a number of species. Furthermore, meristem regenerated plants usually maintain the genetic characteristics of the parent plant (11). Micropropagated strawberry plants were comparatively better in different characters (crown size, number of runners, flowering time and yield of berries) than conventionally propagated runner plants (12, 13).

Runner tip explants have been widely used for shoot induction and multiplication in many strawberry cultivars using modified MS and Knops media (14, 15). An *in vitro* production of plants involves the application of plant growth regulator such as auxin and cytokinins for initiation process. The standardization of protocol and procedure of micropropagation of strawberry was successfully attempted by many (3, 16, 17). Morozova (18) reported high concentration of BAP is the best, while other authors suggested IAA + BAP + GA3

(19) and BA + IBA (20) for strawberry micropropagation. The maximum degree for the amount of branches of 5.2 was recorded when explants cultured in MS with 0.5 mg / l IBA + 0.1 mg /l BA (21). Borkowska (20) applied lower concentrations (0.5 ppm BA and 0.1 ppm IBA) for growing plantlets. In this paper a simple and rapid protocol for direct regeneration from runner tip explant using different concentrations of growth regulators is reported.

Materials and Methods

The runner tips measuring about 0.4 cm long were used from healthy runners of strawberry and used as explants for the present investigation were collected from the greenhouse.

Sterilization of plant material : The explants were collected in clean polythene bags and brought to the laboratory. They were cut into convenient sizes and rinsed thoroughly in running tap water for 10 minutes. The explants were surface sterilized with 3-4 drops of teepol for 10 minutes then citric acid (0.4%) and ascorbic acid (0.2%) for 10 minutes followed by bavistin (0.4%) and streptocycline (0.3 - 0.4%) for 90 minutes to remove of any systemic contamination. Finally, the explants were surface sterilized with mercuric chloride (0.1%) for 5 minutes inside the laminar flow cabinet. The sterilant was then washed with double distilled water for 5-6 times under aseptic condition and the material was ready for inoculation on appropriate nutrient medium.

Media for micropropagation : MS basal media supplemented with different concentrations and combinations of growth regulator were prepared as a media for micro-propagation (22).

Media for shoot proliferation : For shoot proliferation sterilized explants was cultured on MS basal media supplemented along with IAA, BAP and kinetin (Table 1) and data were recorded on time taken for shoot multiplication and number of shoots obtained from each culture.

Media for root induction : For rooting, regenerated shoots were sub-cultured in solidified MS medium supplemented along with concentration of IBA or NAA and their combinations (Table 2) and data were recorded on time taken for root initiation, number of shoot showing root formation, number of roots, length of roots and days required to transplantation.

Transplantation : Rooted plantlets were taken out from culture bottles and washed thoroughly with tap water to remove the culture medium from the roots. Washed plantlets were sprayed with bavistin (0.1%) and planted to soil and various soilless media (cocopeat + perlite + vermicompost- 3:1:1, 4:0:1, 4:1:0, 4:1:1 and 4:1:2) in PVC pots and transferred in culture room for hardening.

Table 1: Combination and concentration of plant growth regulators added in MS basal media in order to induce shoot proliferation in runner tip explant of strawberry

Shoot proliferation media code	Basal media	Combination and concentration of growth regulators (µM)		
		IAA	BAP	Kinetin
SM_1	MS	-	0.5	-
SM_2	MS	-	1.0	-
SM_3	MS	0.1	0.5	-
SM_4	MS	0.5	1.0	-
SM_5	MS	0.1	-	2.0
SM_6	MS	0.5	-	2.5
SM_7 (control)	MS	-	-	-

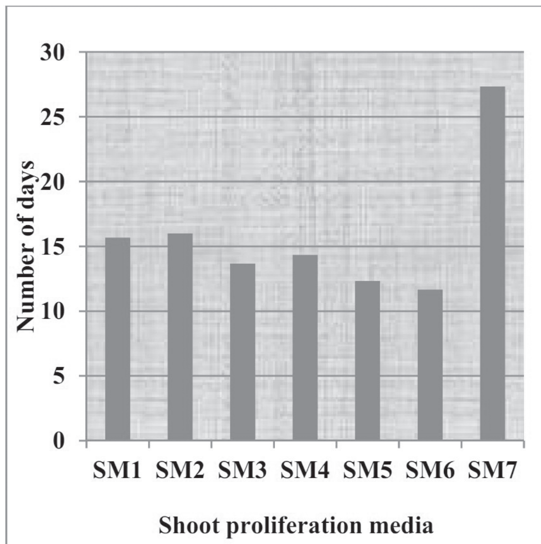

Figure1: Time taken for shoot proliferation

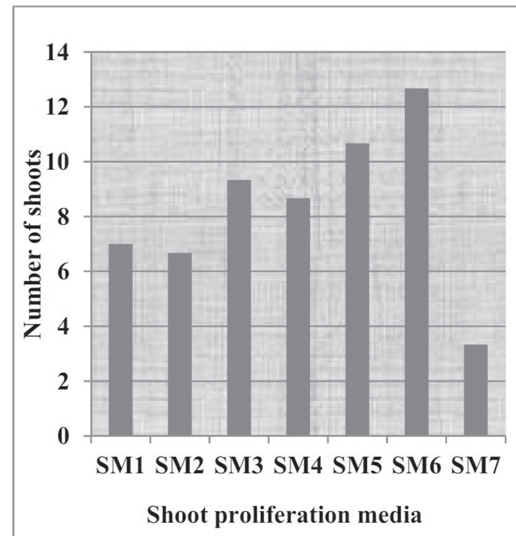

Figure 2: Number of shoot/ culture

Fig. 1 & 2: Effect of different media on shoot proliferation from runner tip explants of strawberry cv. Camarosa

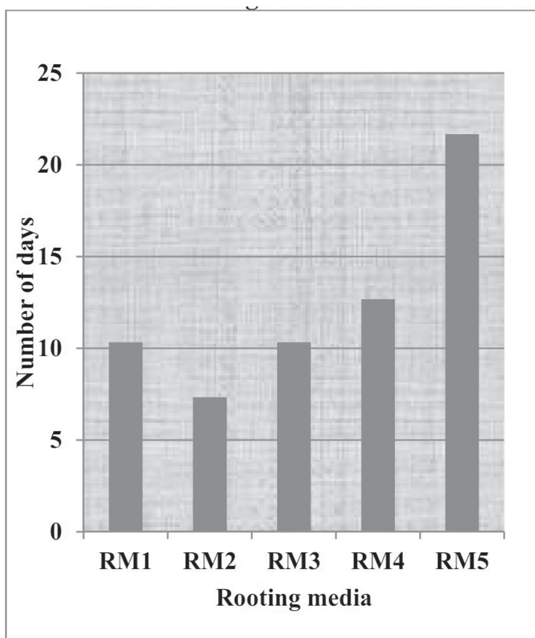

Figure 3: Time taken for root initiation (days)

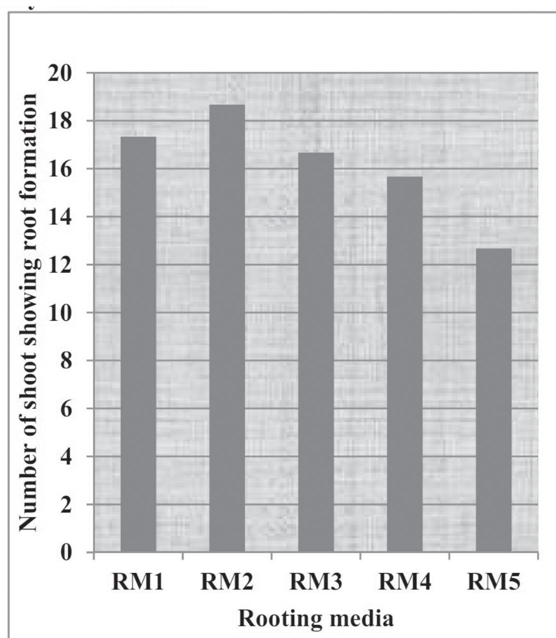

Figure 4: Number of shoot showing root formation

Fig. 3 & 4 : Effect of different rooting media on time taken for root initiation and number of shoot showing root formation in strawberry cv. Camarosa

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Table 2: Combination and concentration of plant growth regulators added in MS basal media for rooting in strawberry

Rooting media code	Basal media	Combination and concentration of growth regulators (mg/l)	
		IBA	NAA
RM_1	MS	1	-
RM_2	MS	2	-
RM_3	MS	-	1
RM_4	MS	-	2
RM_5 (control)	MS	-	-

Results and Discussion

Shoot proliferation : The data obtained on the shoot proliferation from runner tip explants in MS medium supplemented with different levels of auxins and cytokinins either alone or in combination are presented in Figure 1 and 2 for strawberry cultivar Camarosa. The data in Figure 1 revealed that the minimum number of days taken for shoot proliferation (11.67) was recorded in SM_6 (MS + 0.5 μM IAA + 2.5 μM Kinetin) followed by SM_5 (12.33) and SM_3 (13.67) while maximum number of days (27.33) for shoot proliferation was recorded in SM_7 (MS basal + no hormone) followed by SM_2 (16.00) and SM_1 (15.67). The highest number of usable shoots was observed (Figure 2) at the concentration of SM_6 (MS + 0.5 μM IAA + 2.5 μM kinetin) that was 12.67 followed by SM_5 (10.67). Whereas, minimum shoots per culture (3.33) were reported in control (MS basal + no hormone) followed by SM_2 (6.67)

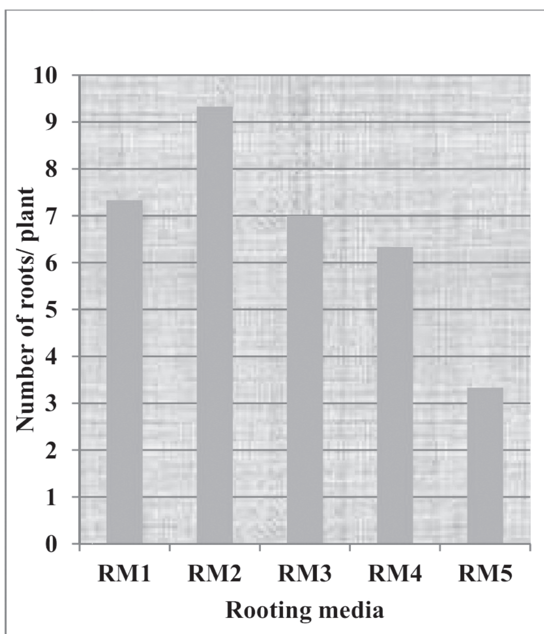

Figure 5: Number of roots/ plant

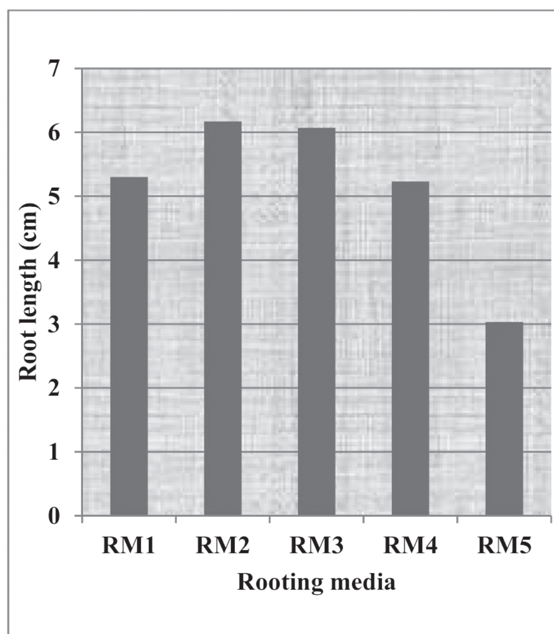

Figure 6: Length of roots (cm)

Fig. 5 & 6: Effect of different rooting media on number of roots/ plant and length of roots (cm) of strawberry cv. Camarosa

treatment. This difference may be attributed by the difference of hormones and genetic makeup or physiological condition of the explants. Rapid shoot regeneration in strawberry using MS medium containing kinetin or in combination with BA has been reported to be due to differences in endogenous hormone concentrations (15, 23). The highest response of shoot multiplication was obtained in MS medium containing 2.0 mg/ l Kinetin + 0.5 mg/ l IAA for strawberry cv. Ofra using shoot tip explants (24).

Induction of rooting : The data on the effect of different concentration and combination of hormones in MS media on time taken for root initiation and number of shoot showing root formation are presented in Figure 3&4. The data in Figure 3 revealed that the minimum number of days for root initiation (7.33) was recorded in treatment MS + IBA (2 mg/ l) followed by MS + IBA (1 mg/ l) and MS + NAA (1 mg/ l) (10.33) while, maximum number of days taken for root initiation (21.67) was recorded in MS basal alone followed by MS + NAA (2mg/ l) (12.67). It is clear from the data given Figure 4 that various treatments significantly affect the number of shoot showing root formation. Maximum number of shoots which shows root formation (18.67) was recorded in MS + IBA (2 mg/ l) followed by RM_1 (17.33) and RM_3 (16.67) whereas, minimum number of shoots showing root formation (12.67) were recorded in MS + no hormone, followed by RM_4 (15.67).

It is evident from the data (Figure 5) the maximum number of roots (9.33) were observed in MS + IBA (2 mg/ l) followed by (7.33) MS + IBA (1 mg/ l), while minimum number of roots (3.33) were recorded from MS basal alone followed by RM_4 (6.33). The data in figure 6 exhibited an increased length of roots in all MS media treatments over control. Increase in length of roots (6.17 cm) was recorded in MS + IBA (2 mg/ l) whereas, decreased in length of roots (3.03 cm) was recorded in MS basal alone followed by MS + 2 mg/ l NAA (5.23 cm). It is evident Figure 7 that hormone treatments in MS media significantly affect the days ready to transplant. Minimum days ready to transplant (20.33) was recorded in treatment MS + IBA (2 mg/ l) followed by treatment MS + 1 mg/ l NAA (21.67) and maximum days ready to transplant (37.33) were recorded in treatment MS basal with control followed by RM_4 (27.33). The variation may be due to the interaction of hormone concentration and physiology of the explant. The present findings are in conformity with the findings of Haddadi *et al.* (25) reported the highest length of root was produced in MS medium with IBA (1 µM). Mir *et al.* (26) recorded maximal number and increased length of roots on MS medium supplemented with 2 mg/ l IBA. Similar increase in the root length and number of roots was reported by Rai *et al.* (24) from MS medium containing 1.0 mg/ l IBA.

It is clearly evident that form Figure 8 all soilless media showed better effect on survival percent compared to the soil. The maximum survival percentage (94.33%) was rerecorded from the plants grown in S_1 (cocopeat + perlite + vermicompost, 3:1:1) followed by S_5 (89.33) and S_4 (85.67%). Whereas, minimal survival per cent (63.00%) was observed from soil followed by S_3 (74.33%). This is may be due to the effect of substrate influence in better exchange of elements especially cations inside the substrate and they distribute humidity properly around the root zone and it is finally effective in root system and survival of plants. Addition of sand with vermicompost decreased the total porosity, easily available water and total water holding capacity (27). Rai *et al.* (24) also reported better survival percent of strawberry plantlets in soilless (cocopeat + perlite + vermiculite, 3:1:1) substrate.

Conclusion

Results of mass multiplication of strawberry study revealed that large number of high quality plant material can be achieved in a very short period of time. The planting material derived through tissue culture techniques using runner tip as explant, show uniformity and also the planting material will have the least chances of viral and other disease infestation. In the experiment plants were cultured on MS media containing 0.5 µM IAA + 2.5 µM kinetin using

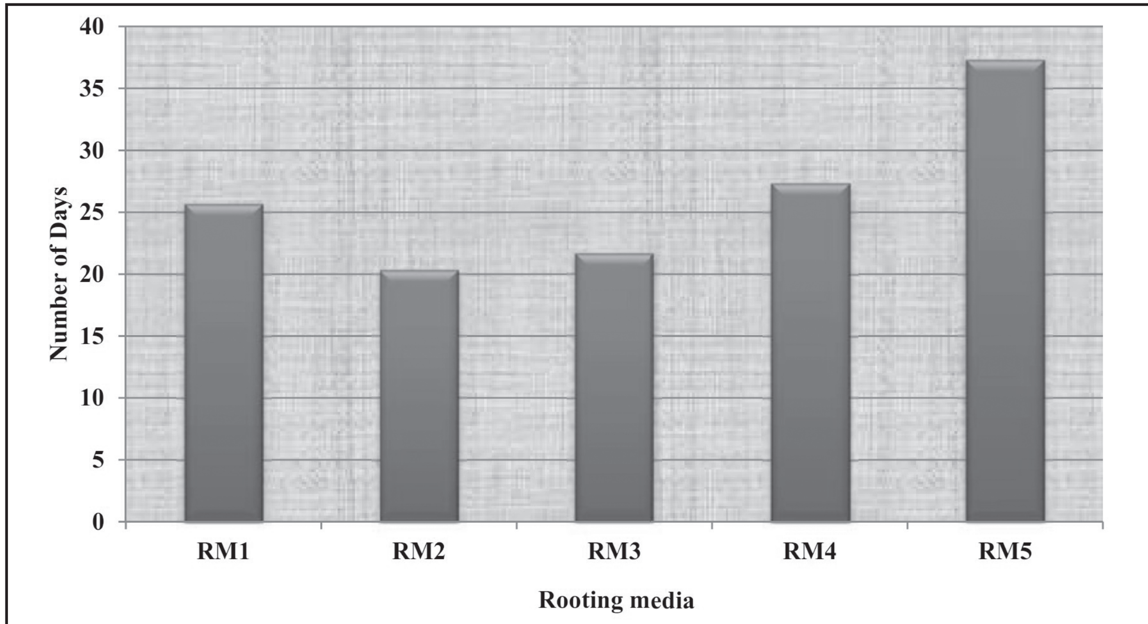

Fig. 7: Effect of different rooting media on days ready to transplant of strawberry cv. Camarosa

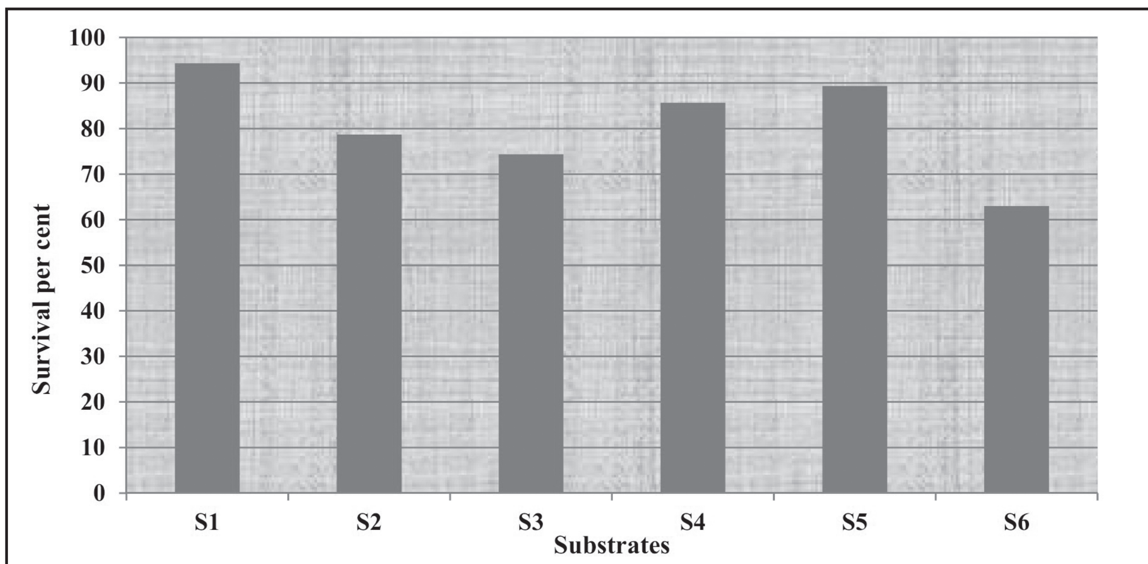

Fig. 8: Effect of different substrates on per cent survival after transplantation in strawberry cv. Camarosa

Vol. 10 (1) 37-45 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

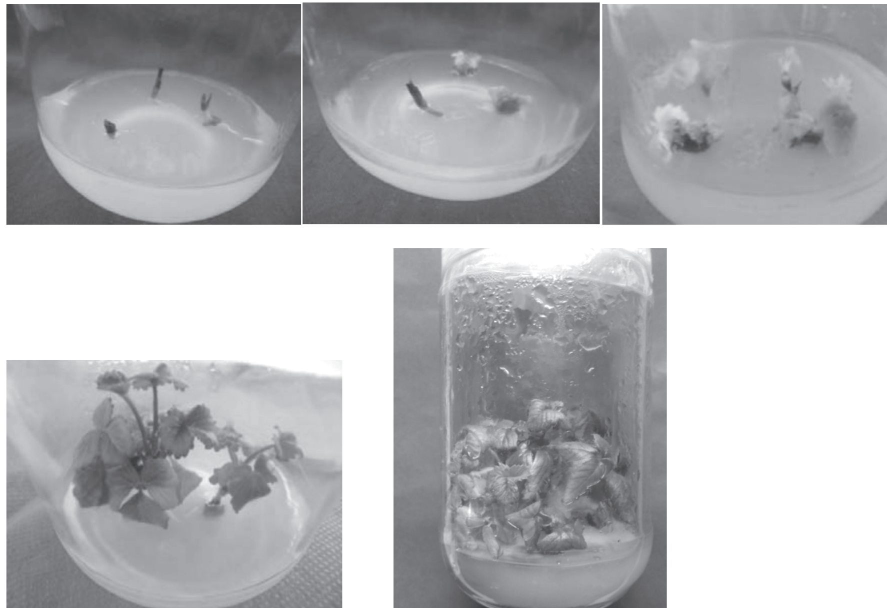

Fig. 9: Different stages of culture for multiple shoot regeneration in strawberry cv. Camarosa from runner tip explants

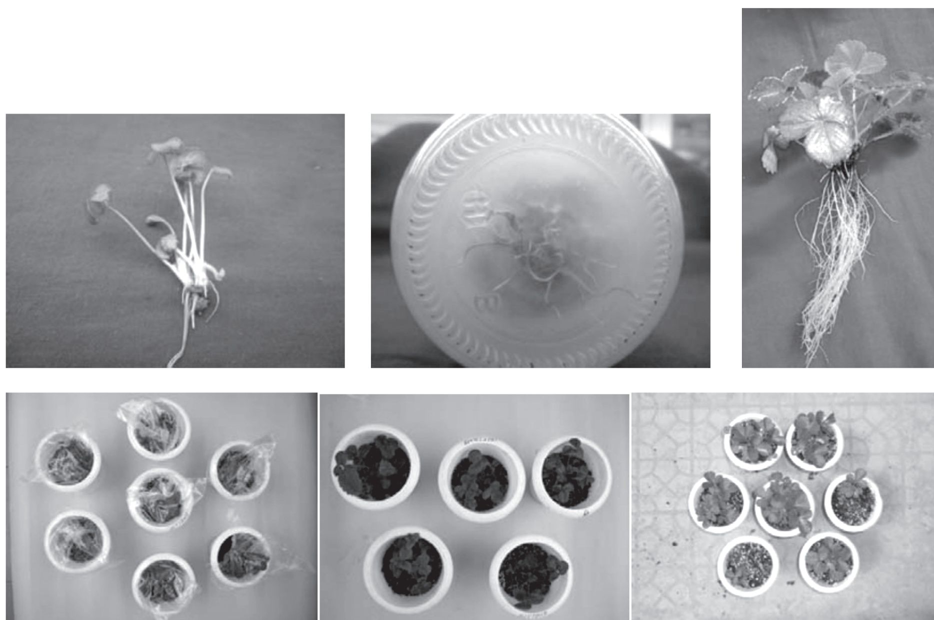

Fig. 10: Different stages of rooting and transplantation of regenerated plants in pots in different media

runner tip as explants take less time for shoot proliferation and produce highest number of shoots/ culture. While, for *in vitro* rooting MS media containing IBA (2 mg/ l) produced better quality roots with less time of root initiation. Plantlets transferred in cocopeat + perlite + vermicompost (3:1:1) reported maximum transplantation success.

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Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) 46-55 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Influence of Sucrose and Plant Growth Regulators on Growth and Forskolin Production in Callus cultures of *Coleus forskohlii*

G. Swaroopa, M. Anuraadha and T. Pullaiah
Department of Botany, Sri Krishnadevraya University,
Anantapur 515003, A.P., India
*For Correspondence - pullaiah.thammineni@gmail.com

Abstract

An efficient protocol for callus culture, suspesion culture and forskolin production in *Coleus forskohlii* has been described. Best explant for callus induction was leaf and best medium was MS medium. Of all the concentrations tried sucrose at 3% and 4% resulted in higher biomass accumulation. The maximum callus biomass production was observed on 2, 4-D supplemented medium at 2mg/l concentration when compared to other auxins and cytokinins. Of all combinations 3mg/l NAA + 1mg/l BAP resulted in highest percentage of callus induction and biomass production. This combination in the medium resulted in high percentage of forskolin, but less biomass compared to 2 mg/l 2, 4-D. Maximum production of forskolin was observed on the 14th day of callus culture while in suspension culture maximum production of forskolin was observed on the 10th day of culture

Key words: Forskolin, *Coleus forskohlii*, callus cultures, suspension cultures, secondary metabolites.

Introduction

Coleus forskohlii (Willd.) Briq. is a perennial herb of the family Lamiaceae. The fasciculated tubers of *C. forskohlii* on drying yield a unique labdane diterpenoid called forskolin. The plant is valued, as forskolin drug is used for the treatment of glaucoma, congestive cardiomyopathy and asthma (1). Forskolin possesses positive inotropic and blood pressure-lowering activities through intra venous administration in a central nervous system (CNS) depressant, bronchodilator (2) serves nerve regeneration and lowers intra ocular pressure (3). Owing to its unique pharmacological properties and its potential, the plant has attracted worldwide attention in recent years. It is only source of this compound detected so far. With this background, the growing demand of this forskolin as a unique research tool and its potential as a therapeutic agent resulted in interest to raise protocols for richer sources of forskolin, i.e., production through cell or organ cultures. The production of forskolin by cell and callus cultures might become a further source for stable production of forskolin besides field grown plants. Hence the present investigation has been undertaken.

Materials and Methods

Initiation and Maintenance of callus Culture : Different explants from *in vitro* grown plantlets such as root, leaf, petiole, internode and node were excised and inoculated on MS medium, B_5 medium and LS medium fortified with 2 mg/l 2,4-D . After 30 days observations were made and recorded. Best suitable explant screened in the above experiment was inoculated on to MS medium supplemented with concentrations of different growth regulators individually and in combination. Observations were recorded after 1 month. The main objective of this experiment is to study the effect of growth regulators on nature of response, percentage of response and variations in forskolin content. The callus grown on the best optimized medium was allowed to

grow for 3 weeks and the callus was maintained by regular subculturing at 3-week intervals, on the same medium. The biomass was estimated by determining fresh and dry weights of the callus at regular intervals. For dry weight determination, the cells were dried in a hot air oven at 60ºC for 24 hours and weighed.

Initiation and Maintenance of Suspension Culture : MS liquid medium supplemented with 3 mg/l NAA and 1 mg/l BAP was prepared. Media pH was adjusted 5.7 before autoclaving. About 50 ml of liquid medium was dispensed into 250 ml Erlenmeyer flasks. Friable callus weighing approximately 10 gm was transferred to the flasks containing medium. Cultures were incubated on a rotary shaker at 120 rpm in darkness and temperature was maintained at 25±2°C. The cell suspensions were filtered through appropriate sterile sieves to obtain single cells and few celled aggregates which were used as inoculum for subculture. Fine cell suspensions were obtained by repeated subculturing of callus and removing small clumps at every stage. The cell suspension cultures were maintained on the same medium by subculturing at 2-week interval.

Results and Discussion

Establishment of Callus cultures : Successful induction of friable callus and its establishment *in vitro* is of great importance. The source of explants for initiation of callus in the present study is from mother cultures established *in vitro*. The nutritional requirements of plant cells and tissues vary from species to species and therefore a number of media have been devised for specific plant and tissue by different workers. Healthy and friable callus is the prerequisite for any secondary metabolite research. To obtain this callus cultures have been initiated from different explants such as petiole, leaf, root, internode, node of *in vitro* germinated plants. These explants were placed on MS. B5 and LS medium supplemented with 2 mg/l 2, 4-D to study the effect of media and explants on callus proliferation (Table 1). Morphogenetic response varied with the type of explant used. The percent callogenic response was high from leaf explants when placed on MS medium (Fig. 1) when compared to B5 and LS medium. Hence in the subsequent experiments of growth studies and establishment of suspension cultures were performed with leaf derived callus. The data subjected to statistical

Table-1 Effect of explant and media type on callus induction and nature of callus *Coleus forskohlii*

Explant (E)	% of Callus Induction (Media)			Nature of Response		
	MS	B5	LS	MS	B5	LS
	Mean ± SD	Mean ± SD	Mean ± SD			
Leaf	83.33 ± 5.8	73.33 ± 5.8	60.00 ± 10.0	+++	++	+
Stem	76.67 ± 5.8	66.67 ± 5.8	43.33 ± 5.8	++	++	++
Node	53.33 ± 11.5	40.00 ± 10.0	23.33 ± 5.8	++	+	++
Internode	50.00 ± 10.0	53.33 ± 5.8	50.00 ± 10.0	+	+	+
Root	33.33 ± 5.8	23.33 ± 5.8	20.00 ± 10.0	+	+	+
	Explant (E)	Media(M)	Interaction(E*M)			
F-Value	65.65*	16.34*	0.44NS			
SE	2.509	1.940	4.53			
CD(5 %)	6.245	6.295	----			

* Significant at 5 % Level, NS : Non-Significant, Number of Replication=3
+++ = Best ++ = Better + = Good

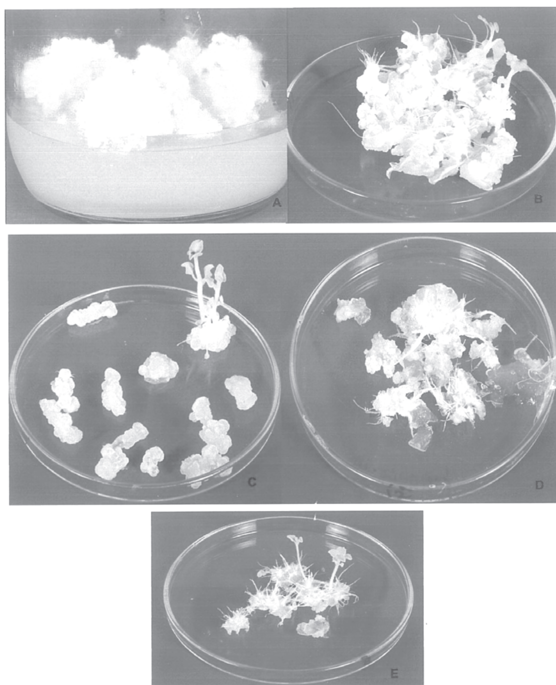

Fig. 1. Induction of callus on MS medium supplemented with 2 mg/l 2,4-D from different explants of *Coleus forskohlii.* A. Leaf, B. Node, C. Intrnode, D. Root, E. Petiole.

analysis indicated the significant difference in percent of response with respect to different media, (F=16.34*) different explants (F=65.65*). The significant feature of the MS medium is its very high concentration of nitrate, potassium and ammonia. The levels of inorganic nutrients in the B_5 medium are lower than in MS medium. Higher, healthier and faster callogenic response may be attributed to this feature of MS medium. Soft creamish, friable callus resulted on MS medium is used to establish suspension cultures. Compact granular callus with less biomass was obtained when the explants were inoculated on B_5 and LS medium. The advantage of leaf being the source of the dedifferentiation and callus formation in *Corydalis saxicola* and *Saussurea obvallata* was also confirmed (4, 5). The differences in cultural requirements exist among different parts of the same plant may be attributed to the various levels of endogenous plant growth regulators of explants from different positions (6).

Effect of Sucrose on Callus Induction and Forskolin Content : It is assumed that sucrose is the best carbohydrate in cell culture media because it is the main transport form of carbohydrates in most species (7, 8). The effect of different concentrations of sucrose on biomass yield and forskolin content was tested. Effect of sucrose not only influenced the biomass, nature

Table-2 Effect of Sucrose concentration on callus biomass production, nature of response and forskolin content

Sucrose concentration	Bio mass (gm) Fresh wt Mean ± SD	Forskolin content (mg/kg DCW) Mean ± SD	Nature of response
2 %	82.07 ± 2.1	173.1 ± 2.1	Friable callus
3 %	86.07 ± 2.1	182.0 ± 2.0	Soft, Creamish, friable callus
4 %	89.07 ± 2.1	186.0 ± 2.0	Friable callus with rhizogenesis
5 %	85.07 ± 2.1	176.1 ± 2.1	Compact, nodular callus with
6 %	83.07 ± 2.1	172.0 ± 2.0	rhizogenesis
F-Value	5.10*	25.25*	
SE	1.212	1.196	
CD at 5 %	3.819	3.768	

* Significant at 5 % Level, Number of Replication

of the callus but also affected the forskolin content (Table 2). Of all the concentrations tried sucrose at 3% and 4% resulted in higher biomass accumulation i.e. 86gm and 89gm (Fresh wt) within 15 days of initiation. However maximum forskolin content was recorded in callus proliferated on medium fortified with 4% sucrose. The higher levels of forskolin at 4% sucrose might be due to rhizogenic callus. As the basic feature of *Coleus forskohlii* is the presence of forskolin in roots and not in any other part. However the main objective of the present study is to induce callus and suspension cultures for the production of forskolin. In the above experiment only 3% sucrose concentration is preferred as it is resulting in only friable callus without organogenesis.

The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. The maximum yield of rosmarinic acid produced by cell suspension cultures of *Salvia officinalis* was 3.5 g/l when 5% of sucrose was used but it was 0.7 g/l in the medium containing 3% sucrose (9). The accumulation of rosmarinic acid in suspension culture cells of *Coleus blumei* can be stimulated by higher sucrose content in the medium (10, 11, 12). Since sucrose has always been the carbon source of choice, a concentration range from 2 to 6% was assayed. Sometimes unorganized cells become organized and developed shoots or roots under controlled conditions of plant growth regulator. To develop organ, starch accumulation is the perequisite and it may work as a catalyst in the cell to develop shoots and roots (13). In this study higher level of sucrose resulted in rhizogenic callus and increase in forskolin content. The biomass production was high at 4% (89.07 ± 2.1gm fw) but the forskolin content was high at 3% (186.0 ± 2.0mg/kg DCW). The data subjected for statistical analsis indicate the significant difference to varied sucrose concentrations in terms of biomass production (F=5.10*) and forskolin content (F=25.25*). Morphological variations of callus cultures of *Coleus forskohlii* on medium supplemented with different concentrations of sucrose is shown in Fig. 2.

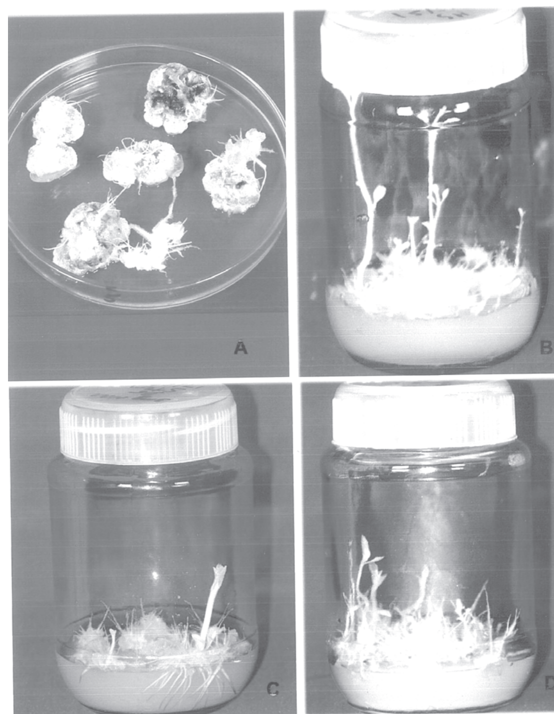

Fig. 2. Callus induction on various concentrations of sucrose. A. 3% Sucrose, B. 4% Sucrose, C. 5% Sucrose, D. 6% Sucrose.

Effect of Plant Growth Regulators : There is a vast amount of literature on the effects of plant growth regulators on secondary metabolism in cultured plant cells (14, 15, 16). After screening the best medium and explant in order to study the effect of plant growth regulators (PGRs) on the callus culture different PGRs were tried at different concentration and various combinations. MS medium lacking growth regulator served as control. The data on callus formation was recorded and expressed as percentage of frequency. Based on the results the highest percentage of callus induction was obtained on MS medium individually amended plant growth regulator such as 2, 4-D at 2 mg/l (Table 3) followed by NAA and IAA. Rhizogenesis on callus was observed on NAA and IAA supplemented

Table-3. Effect of plant growth regulators on percentage of callus proliferation and biomass production

Plant growth regulator	Conc (mg)	% of callus proliferation Mean ± SD	Fresh Wt Mean ± SD	Dry Wt Mean ± SD
2,4-D	1	53.3 ± 5.8	151.2 ± 1.1	7.89 ± 0.1
	2	76.7 ± 5.8	182.6 ± 1.2	8.98 ± 0.1
	3	60.0 ± 0.0	147.5 ± 1.1	7.07 ± 0.1
	4	40.0 ± 10.0	96.6 ± 1.2	5.80 ± 0.1
	5	26.7 ± 15.3	66.4 ± 1.2	4.10 ± 0.1
NAA	1	53.3 ±11.5	55.5 ± 1.0	3.89 ± 0.2
	2	63.3 ± 5.8	80.2 ± 1.0	5.18 ± 0.1
	3	76.7 ± 5.8	89.0 ± 1.0	5.92 ± 0.1
	4	60.0 ± 0.0	80.2 ± 1.0	5.15 ± 0.2
	5	40.0 ± 10.0	76.1 ± 1.0	4.56 ± 0.2
IAA	1	53.3 ±5.8	27.24 ± 2.0	1.60 ± 0.1
	2	60.0 ± 10.0	56.77 ± 2.0	2.50 ± 0.1
	3	33.3 ± 5.8	39.68 ± 2.0	1.76 ± 0.1
	4	20.0 ± 10.0	19.81 ± 2.0	1.04 ± 0.1
	5	10.0 ± 0.0	18.01 ± 2.0	0.79 ± 0.1
BAP	1	66.7 ± 5.8	21.1 ± 0.1	1.21 ± 0.02
	2	46.7 ± 5.8	26.5 ± 0.1	1.57 ± 0.02
	3	40.0 ± 10.0	33.5 ± 0.1	1.75 ± 0.02
	4	36.7 ± 15.3	32.1 ± 0.1	1.51 ± 0.02
	5	33.3 ± 5.8	30.1 ± 0.1	1.44 ± 0.02
KN	1	40.0 ± 10.0	29.1 ± 0.5	1.50 ± 0.3
	2	56.7 ± 15.3	31.7 ± 0.5	1.64 ± 0.4
	3	50.0 ± 10.0	34.1 ± 0.5	1.74 ± 0.4
	4	33.3 ± 5.8	37.3 ± 0.5	1.88 ± 0.3
	5	30.0 ± 17.3	36.5 ± 0.5	1.91 ± 0.5
F-Value		5.180*	0.328*	0.226*
SE		3.3671	8.9163	0.4766
CD (5%)		0.005	0.856	0.921

*Significant at 5% level, Number of Replication=3

medium. The cytokinins, BAP and KN at various concentrations and combinations were also tested. Callus on 2, 4-D supplemented medium was well proliferated creamish, spongy and loosely arranged. The maximum callus biomass production was observed on 2, 4-D supplemented medium (182.6 ± 1.2 gm) at 2mg/l concentration when compared to other auxins and cytokinins. The moisture content of callus induced on medium supplemented with 2, 4-D was high as compared to other auxins supplemented media resulting in low dry matter yield. In NAA supplemented medium, the callus was green in color and hard and granular. But in

IAA supplemented media the callus was solid in nature and green in color. Yield of fresh biomass was high on medium with 2, 4-D (182.6 ± 1.2 gm fw and 8.98 g DW). Morphological nature of calli on this medium is healthier when compared to other media but the forskolin content was negligible. The data subjected for statistical F-test indicate a significant difference in cultures subjected to various hormones in terms of callus induction, 2,4-D (F=5.33*), NAA (F=7.08*), IAA (F=20.26*), BAP (F=5.30*), KN (F=5.9*). Phytohormones such as auxins and cytokinins have shown the most remarkable effects on growth and productivity of plant metabolites. In general, an increase of auxin levels, such as 2, 4-D in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites. Hence auxins are added at a low concentration or omitted during production of metabolites. Decendit reported that cytokinins stimulated alkaloid synthesis after withdrawing auxin from the medium in cell line of *Catharanthus roseus*. However productions of Ubiquinone-10 by *Nicotiana tabacum* (17) and diosgenin by *Dioscorea deltoidea* (18) were stimulated by high levels of 2, 4-D. Cytokinins have also been reported to stimulate alkaloid biosynthesis from different plant cell tissue (19, 20, 21). The combination of different growth regulators at different concentrations were also tested for callus induction (Table 4). Of all combinations 3mg/l NAA + 1mg/l BAP resulted in highest percentage of callus induction (60.7 ± 5.8) and biomass production (89.5 ± 1.0). The combination in the medium resulted in high percentage of forskolin (184 ± 5.0 mg/kg DCW) (Table 5), but less biomass compared to 2 mg/l 2, 4-D. However there was significant difference in response to different growth regulators when used individually and in combination in terms of forskolin content (F=463.81*). But in the present study the main objective is to induce callus and suspension cultures. Hence 2mg/l 2, 4-D is preferred in this experiment as it is resulting in friable callus. In case of the elicitation studies, i.e enhancement of forskolin by using elicitors in suspension cultures, the combination of 3mg/l NAA+ 1mg/l BAP has been used as it has resulted in high percentage of forskolin. High concentrations of auxins and cytokinins hindered the frequency of callusing and biomass content. Lower levels of auxin induced callus, while higher levels resulted in rhizogenic callus. Beyond 3 mg/l the percent frequency of callus induction was not very significant. Although several authors demonstrated that 2, 4-D was necessary to initiate callus induction (22) it was ineffective in our experiences to produce forskolin. Callus proliferation on MS medium supplemented with various plant growth regulators individually and in combination is depicted in Fig. 3.

Growth and Forskolin Profile of Callus Cultures and Suspension Cultures : Because knowledge of the growth characteristics of a

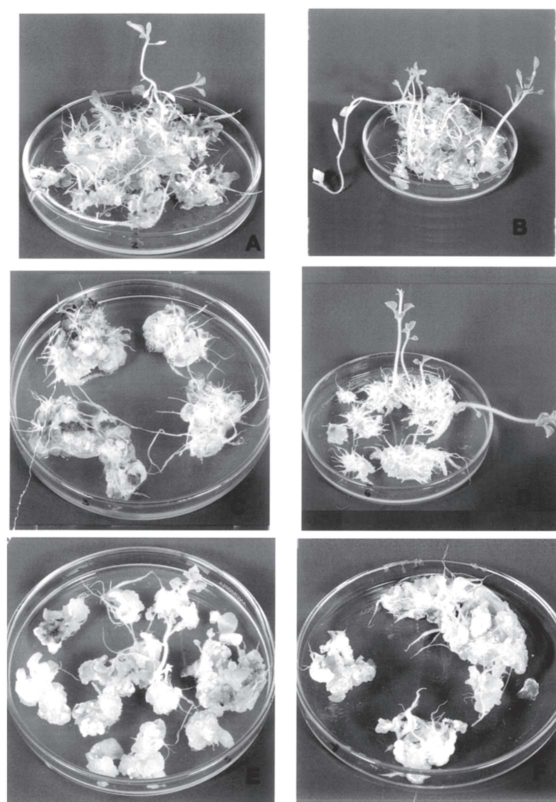

Fig. 3. Induction of callus on MS medium supplemented with various plant growth regulatores individually and in combination. A. BAP, B. KN, C. IAA, D. NAA, E. IAA + BAP, F. NAA + BAP.

Table-4. Influence of different combinations of plant growth regulators on callus biomass of *Coleus forskohlii*

Treatment	Concentration			No. of explants (%) showing Callus	Bio mass (gm)	
					Fresh weight	Dry weight
	IAA	NAA	BAP	Mean ± SD	Mean ± SD	Mean ± SD
GC1	0.5	-	1.0	40.0 ± 0.0	60.4 ± 1.0	3.4 ± 0.1
GC2	1.0	-	1.0	40.0 ± 10.0	70.5 ± 1.0	3.9 ± 0.1
GC3	1.5	-	1.0	53.3 ± 5.8	75.8 ± 1.0	4.2 ± 0.1
GC4	2.0	-	1.0	53.3 ± 15.3	60.2 ± 1.0	3.1 ± 0.1
GC5	2.5	-	1.0	36.7 ± 5.8	59.6 ± 1.0	2.9 ± 0.1
GC6	3.0	-	1.0	33.3 ± 11.5	56.1 ± 1.0	2.5 ± 0.1
GC7	-	0.5	1.0	46.7 ± 5.8	60.8 ± 1.0	4.2 ± 0.1
GC8	-	1.0	1.0	53.3 ± 11.5	78.4 ± 1.0	4.9 ± 0.1
GC9	-	1.5	1.0	56.7 ± 5.8	79.8 ± 1.0	5.1 ± 0.1
GC10	-	2.0	1.0	56.7 ± 15.3	82.5 ± 1.0	5.0 ± 0.1
GC11	-	2.5	1.0	50.0 ±17.3	84.2 ± 1.0	5.3 ± 0.1
GC12	-	3.0	1.0	60.7 ± 5.8	89.5 ± 1.0	5.7 ± 0.1
F-Value				2.65*	404.78*	67.53*
SE				6.000	0.577	0.054
CD (5 %)				17.514	1.684	0.158

* Significant at 5 % Level, Number of Replication=3

Table-5 Effect of different plant growth regulators on Forskolin content

Hormones	Concentration (mg/l)	Forskolin content (mg/kg DCW) Mean ± SD
2,4 –D	2	0±0.0
NAA	2	150 ± 5.0
IAA	2	115 ± 5.0
BAP	2	124 ± 5.0
KN	2	100 ± 5.0
IAA + BAP	3+1	134 ± 5.0
NAA + BAP	3+1	184 ± 5.0
F-Value		463.81*
SE		2.671
CD at 5 %		8.102

* Significant at 5 % Level, Number of Replication=3

given suspension cell culture is important in bioprocess engineering and manipulating secondary metabolite production, growth kinetic parameters were first determined. A time course study of growth of callus cultures were conducted for 30 days on MS medium fortified with 2 mg/l 2, 4-D and 3% sucrose showed a sigmoidal curve. Observations were made from the second day after incubation and up to the 30th day with 2 days intervals and callus biomass was recorded and presented in the Table 6. The callus biomass was expressed as fresh weight and dry weight (g/kg). The fresh biomass was high in callus cultured on solid medium. The growth cycle showed a lag phase of 4 days followed by an exponential phase of growth which resulted in increased growth till the 22nd day (39.73 ± 1.9 gm FW) of culture and thereafter it reached the stationary phase. The culture remained green up to 24 days and then started browning showing symptoms of death phase. Three replications were used for each experiment. Forskolin content was monitored throughout the culture period. Forskolin levels decreased soon after inoculation up to 6th day of incubation. However, from the sixth day onwards the content increased rapidly for 20 days, keeping in pace with exponential phase of growth. The forskolin content did not enhance during the post exponential phase of growth. Thus maximum production of forskolin was observed on the 14th day of culture which was about 115.27 ± 3.1 mg/kg dry weight of biomass. However, growth rate of the cultures may vary from species to species and even cell line to line (23, 24).

Table-6. Growth studies of callus cultures of *Coleus forskohlii*

Number of days	Fresh weight of Callus	Forskolin content (mg/kg DCW)
	Mean ± SD	Mean ± SD
0	5.90 ± 1.0	62.17 ± 2.1
2	7.26 ± 1.0	65.23 ± 1.2
4	9.69 ± 1.0	72.07 ± 1.9
6	12.13 ± 1.1	77.17 ± 2.1
8	16.67 ± 1.0	85.17 ± 2.9
10	21.50 ± 1.1	89.17 ± 2.1
12	24.70 ± 1.9	104.37 ± 4.1
14	27.37 ± 0.8	115.27 ± 3.1
16	30.50 ± 1.3	101.27 ± 2.2
18	34.87 ± 0.9	91.27 ± 2.2
20	37.23 ± 1.0	85.27 ± 2.2
22	39.73 ± 1.9	84.13 ± 2.0
24	38.23 ± 2.1	77.17 ± 2.1
26	37.57 ± 1.9	75.50 ± 2.6
28	35.47 ± 3.1	70.17 ± 2.1
30	33.67 ± 2.1	70.13 ± 3.0
F-Value	176*	52*
SE	0.907	2.56
CD at 5 %	2.514	5.64

* Significant at 5 % Level,
Number of Replication=3

The growth study of suspension cultures were also conducted for 16 days which exhibited a sigmoidal curve. Observations were made from the second day after incubation and continued upto the 16th day with 2 days intervals and cell biomass data was recorded and tabulated (Table 7). The maximum average biomass yield was observed from fifth day to ninth day after that the biomass production decreased and the pale yellowish green color of biomass turned into pale brownish color. Cell suspension growth was determined by packed cell volume (PCV) at 2-day intervals. Three replications were used for each experiment. Forskolin content was also monitored throughout the culture period. Forskolin levels decreased soon after inoculation upto sixth day of incubation. However from the sixth day onwards the content was increased. Thus maximum production of forskolin was observed on the 10th day of culture which was about (89.50 ± 4.6 mg/kg) of the dry weight of biomass. In suspension cultures the packed cell volume was high (17 ml/20 ml) on eighth day of

Table-7. Growth studies of Suspension cultures of *Coleus forskohlii*

Number of days	Packed cell volume/20ml	Forskolin content (mg/kg DCW)
		Mean ± SD
0	5 ml/l	72.17 ± 2.1
2	7 ml/l	75.13 ± 1.1
4	11 ml/l	80.27 ± 2.2
6	15 ml/l	86.23 ± 3.1
8	17 ml/l	87.27 ± 2.2
10	16 ml/l	89.50 ± 4.6
12	14 ml/l	70.27 ± 2.2
14	12 ml/l	71.20 ± 2.1
16	11 ml/l	69.27 ± 4.2
F-Value		24.00*
SE		1.638
CD at 5 %		4.867

* Significant at 5 % Level,
Number of Replication=3

the culture. So, forskolin content was low in the cells growing quickly and initiated from highly friable callus and was more in the cells showing moderate growth and initiated from less friable callus. This implies that secondary metabolite synthesis occurs maximally in relatively slow growing, less friable cells in *C. forskohlii*. Similar results has also been obtained for a number of Solanaceous species, e.g. the fast growing and friable calli or suspended cells in *Capsicum frutescens* accumulated lowest levels of alkaloids as compared to compact and deeply pigmented calli or suspended cells (25). In relatively fast growing and friable cell suspension cultures of *Datura innoxia*, secondary metabolite was lower than slower-growing and more aggregated cultures (25).

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HPTLC Method Development and Validation of Cefpodoxime Proxetil and Ofloxacin in Bulk and Combined Tablet Dosage form

Deepshikha Patle*
Faculty of Pharmaceutical Sciences, PCTE Group of Institutes,
Ludhiana, Punjab, India-141004
*For Correspondence - deepshikha@pcte.edu.in

Abstract

The aim of the study is to develop and validate a simple, precise and rapid high performance thin layer chromatographic (HPTLC) method for Cefpodoxime Proxetil and Ofloxacin in bulk and combined tablet dosage form. Chromatographic separation was accomplished by using precoated silica gel $60F_{254}$ TLC plate (20cm x10cm) with 250µm thickness with mobile phase Chloroform in ratio methanol (9:1 v/v). Detection was performed at isobestic point 255 nm for both drugs. The retesion factor of Cefpodoxime Proxetil and Ofloxacin were found to be 0.63 and 0.25, respectively. The reliability of the method was assessed by evaluation of linearity range (500-3000 ng/spot for both Cefpodoxime proxetil and ofloxacin respectively. Accuracy (99.56 % for Cefpodoxime Proxetil and 99.33% for Ofloxacin), and specificity, in compliance with ICH guidelines.

Keywords: Cefpodoxime proxetil, Ofloxacin, High performance thin layer chromatography (HPTLC), method development, validation.

Introduction

Cefpodoxime proxetil (CEF) (Fig. 1) is chemically, 1-(isopropoxy carbonyloxyl) ethyl (6R, 7R)-7-[2- (2-amino-4- thiazolyl) - (z) - 2-(methoxyimino) acetamido]-3-methoxymethyl-3-cephem-4-carboxylate (1, 11). It comes under broad spectrum third generation cephalosporin antibiotic used in the treatment of upper respiratory tract, urinary tract infections. The drugs official in IP and USP which describe liquid chromatography methods for its estimation. Ofloxacin (OFLO) is a fluoroquinolone derivative. Chemically it is(+/-)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7h-oxo-7H-pyrido-[1,2,3-di]-1,4benzoxazine-6-arboxylic acid (Fig. 2) is mainly used as antibacterial for the treatment of urinary tract infection and sexually transmitted diseases. Literature survey revealed that a number of methods are available for quantitative estimation of Cefpodoxime proxetil and ofloxacin in combination with other drugs. The combination of Ofloxacin (OFLO) and Cefpodoxime proxetil (CEF) is used in the treatment of upper and lower respiratory tract infection and typhoid fever. So far no HPTLC method has been reported for combination of CEF and OFLO by using mobile phase chloroform: methanol (9:1). The aim of present work is to develop a simple, rapid, precise and selective HPTLC method for the estimation of combination of CEF and OFLO (1).

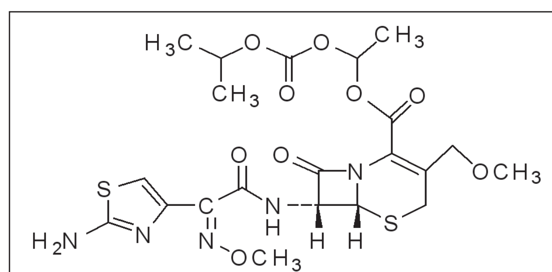

Fig 1: Chemical structure of Cefpodoxime Proxetil

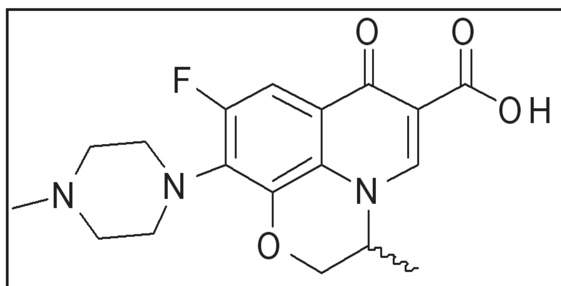

Fig. 2. Chemical structure of Ofloxacin

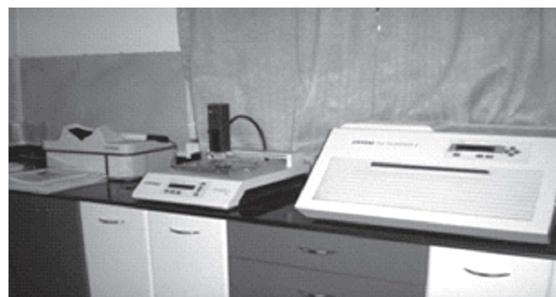

Fig 3. High performance Thin Layer Chromatography

Materials and Methods

***Chemicals and Reagents*:** Pure drugs of CEF and OFLO were obtained from Vashu interprises Pvt. Ltd., Ludhiana as a gift sample. The commercial formulation of CEF and OFLO are available in the ratio of 1:1 (GUDCEF® PLUS) (200/200 mg) as tablets (21). All chemicals and reagents used were of analytical grade and were purchased from Fisher Scientific, India.

***Instrumentation*:** The samples were spotted with a Camag (Fig. 3) 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on silica gel precoated aluminum plate 60 GF254, (20 × 10cm) using a applicator Linomat 5 with software Wincats.

The plates were prewashed by methanol and activated at 110°C for 5 min prior to chromatography. A constant application rate of 0.5ìL/s was employed and the space between two bands was 6 mm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/s scanning speed was employed.

Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (24°C±2) at relative humidity of 60±5%. The length of chromatogram run was 7 cm. Subsequent to the development, HPTLC plates were dried in current of air with the help of air dryer in a wooden chamber with adequate ventilation (2, 5, 6).

Preparation of standard solutions: Accurately weighed 10 mg each of Cefpodoxime proxetil and Ofloxacin were dissolved in 10 ml methanol to obtain 1 mg/ml of stock solution. 1 ml of this stock solution was further diluted with methanol up to 10 ml to obtain working standard solution of 100 µg/ml (3, 4, 17).

***Selection of developing solvent*:** Following solvent systems were tried for the resolution of CEF and OFLO.Initially plane solvents like Methanol, ethyl acetate, Benzene, chloroform, n-hexene were tried. The spots were developed with methanol, n-hexene but no proper resolution observed between CEF and OFLO. Then n-butanol, ethanol and ammonia in the ratio of (6:6:2v/v/v) was tried but again there was no proper resolution obtained. Then chloroform and methanol were tried in the ratio of (7:3v/v) but again there was no proper resolution obtained. Then proportion of chloroform was increased by 2ml which showed good resolution effect. Finally mobile phase used consisted of chloroform: methanol solution (9:1 v/v) which gave good resolution of peaks for CEF and OFLO with no tailing effect [13, 14]. R_f values were found to be 0.25 and 0.63 for OFLO and CEF respectively (12, 15).

***Validation of Method*:** Validation of the optimized HPTLC method was carried out with respect to the following parameters.

Linearity : To establish linearity, the stock solution was applied on the plate using 100 µl

syringe with the help of Linomat V applicator, to give spots of concentrations 500-3000 ng/spot for Cefpodoxime proxetil and ofloxacin. Calibration curve was obtained by plotting peak-area on ordinate and corresponding concentration on abscissa (7, 8).

Method precision: The Intraday and Interday variation of the proposed HPTLC method was assessed using formulation solution. In Intraday study three sets of three concentration solution containing Cefpodoxime proxetil 1000, 1500, 2000 ng and ofloxacin 1000, 1500, 2000 ng were used for study. In Interday study, one set of three concentration solution containing Cefpodoxime proxetil 1000, 1500, 2000 ng and ofloxacin 1000, 1500, 2000 ng were freshly prepared every day for three consecutive days (8, 9). The results are expressed in % RSD. Results are given in table 1.

***Analysis of tablet formulation*:** Formulation of Cefpodoxime proxetil and ofloxacin in combined tablet dosage form (GUDCEF® PLUS-CEF200mg + OFLO200mg) was purchased from local market. Twenty tablets were weighed accurately on balance (Shimadzu AUW-120D) and finely powdered. A portion of the powder equivalent to 10 mg was accurately weighed and transferred to 10 ml volumetric flask from this 1 ml was pipetted out and further diluted to 10 ml to give 100µg/ml of Cefpodoxime proxetil and ofloxacin. From this three bands were applied which had Cefpodoxime proxetil 200 ng/band and ofloxacin 200 ng/band in combination. The TLC plates were then developed and densitometric determination was performed. The peak area of each of the drug was computed and the amount of each drug present in formulation was estimated from the respective calibration curve (7, 8). The results of analysis are depicted in Table 2.

Accuracy: To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed tablet sample solution at three different levels, 80 %, 100 % and 120 %. At each levels of the amount, three determinations were performed (10). Amount of drug present was computed and the results obtained have been depicted in Table 3.

Robustness of the Method: In the robustness study, the influence of small, variations in the analytical parameters on peak area were examined. Three replicates of conc. of 1000ng of Cefpodoxime proxetil and ofloxacin were applied to TLC plate (10). Parameters for the determination of robustness method are given in shown in Table 4 and 5.

***Ruggedness of the method*:** Ruggedness of proposed method was determined by changing the analyst under similar environmental and analytical condition and was expressed in terms of %RSD (16).

Results of ruggedness study are presented in Table 6, 7, 8 and 9.

Specificity of the method: Specificity of proposed method was checked by analyzing the interference of commonly used excipients for its formulation. Pure CFD and OFLO was spiked with common excipients such as talc, Magnesium stearate and starch and then assayed by proposed method (16, 19). It was found that the assay results were unaffected by presence of such excipients and results are reported in Table 10 and 11.

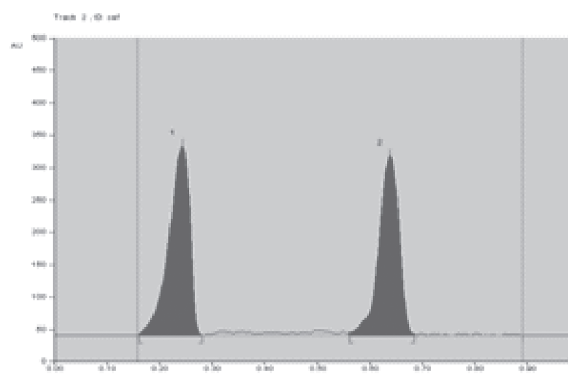

Fig. 4. Densitogram obtained from Ofloxacin (1) and Cefpodoxime proxetil (2)

The identities of the bands for OFLO and CEF were confirmed by comparing the RF and spectra of the bands with those of standards. Typical overlain absorption spectra of OFLO and CEF are shown in Fig 4; 255 nm was selected for densitometric scanning. Peak purity for OFLO and CEF was assessed by comparing the spectra of standards with those acquired at three different points on peaks obtained from the sample (17, 18), i.e. the peak start (S), peak apex (M), and peak end (E) position. 3-D view of CEF and OFLO are shown in Fig 5.

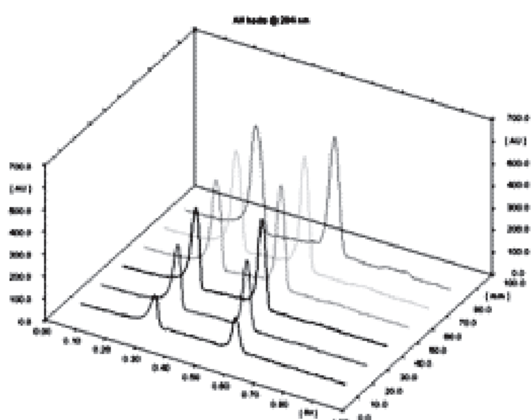

Fig. 5. 3-D view of all tracks at 299(OFLO) and 236 (CEF) nm for Linearity study

Results and Discussion

Method Development of CEF and OFLO : This method was used for augmentation of simultaneous estimation of CEF and OFLO. The mobile phase chloroform in ratio methanol (9:1) (v/v) resulted in good resolution, sharp and symmetrical peaks of CEF at RF 0.25 and OFL at 0.63 respectively. Method was observed that prewashing of silica gel TLC plates with methanol (proceed by drying and activation) and pre-activation of TLC chamber with mobile phase for 30 min assured good repeatability and peak shape of both drugs (20, 23).

Validation of Method

Linearity: The data of linear regression for calibration plots acknowledge good linear relationships between concentration and response over the ranges of 500–3000 ng/spot for both CEF (Fig 6) and OFLO **Fig. 7**. The linear regression equations were $Y = 3.8099X + 2048.7$ ($r^2 = 0.9979$) for CEF and $Y = 3.6676X + 2506.8$ ($r^2 = 0.9971$) for OFLO (22).

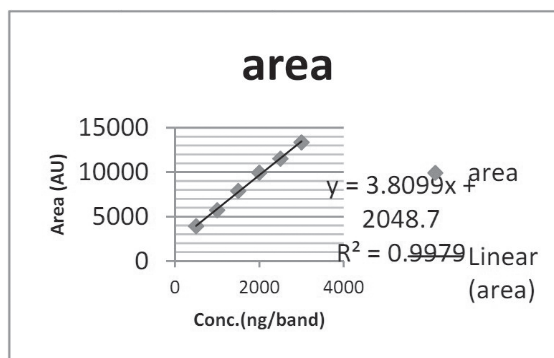

Fig 6. Calibration curve for Cefpodoxime proxetil

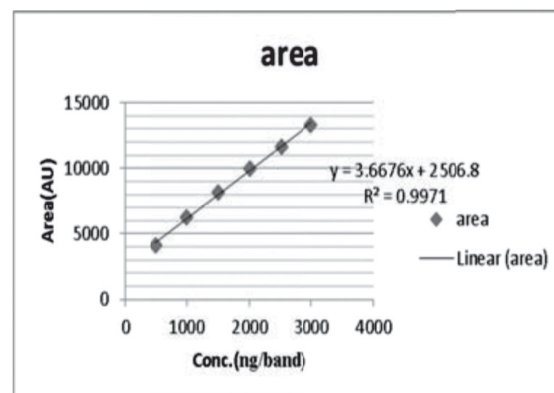

Fig. 7. Calibration curve for ofloxacin

Precision

The method precision revealed relative standard deviation (RSD, %). The results listed in Table 1 shows that method is highly precise.

Conclusion

A simple, linear, accurate, specific and robust HPTLC method has been developed and validated for estimation of CEF and OFLO in their

Table 1. Results of method precision (Intra and Inter day) for Cefpodoxime proxetil and ofloxacin (no. of determinations=3)

Parameters	Cefpodoxime proxetil			Ofloxacin		
Intra day	SD	%RSD	±SE	SD	%RSD	±SE
	2.06	0.03	1.1893	1.20	0.01	0.6928
	1.46	0.01	0.8429	1.22	0.01	0.7043
	1.68	0.01	0.9699	2.02	0.02	1.166
Inter day	0.95	0.07	0.5484	1.88	0.01	1.0854
	2.13	0.02	1.2297	1.22	0.01	0.7043
	1.48	0.02	0.8544	2.20	0.03	1.2701

Table 2. Results of analysis of tablet formulation (no. of determinations=3)

S.No.	Label Claim (ng/band)		Peak Area		Amount Found (ng /band)		% of Label Claim	
	CEF	**OFLO**	**CEF**	**OFLO**	**CEF**	**OFLO**	**CEF**	**OFLO**
1.	200	200	7656.3	7845.5	199.2	198.4	99.6	99.2
2.	200	200	7451.2	7639.6	197.6	197.4	98.8	98.7
3.	200	200	7586.3	7624.5	198.6	199.2	99.3	99.6
			±SD				0.47	0.45
			%RSD				0.407	0.454
			±SE				0.2713	0.2598

SD= Standard deviation, RSD= Relative standard deviation

Table 3. Results of accuracy study of CEF and OFLO (no. of determinations=3)

Lavel of % Recovery	Addition of Tab. Solution	Addition of Standard (API)	Peak Area%		Recovery	
			CEF	**OFLO**	**CEF**	**OFLO**
80%	1500	1200	12561.4	13037.5	99.9	101.0
100%	1500	1500	14029.2	13599.5	101.9	99.0
120%	1500	1800	14959.4	15119.0	99.5	101.7
	SD				1.28	1.40
	%RSD				0.01	0.01
	±SE				0.7390	0.8082

Table 4. Results for robustness study of Cefpodoxime proxetil

S. No.	Parameter Checked	Conc.ng	Area	Mean	SD	%RSD
1.	**Increase saturation time by 30min.**	1000	**CEF** 5677.6	**CEF**	**CEF**	**CEF**
		1000	5603.5	5572.1	1.34	0.01
2.	**Increase saturation distance by 80mm**	1000	5435.3			
		1000	**CEF** 5989.5	**CEF**	**CEF**	**CEF**
		1000	5399.2	5752.8	1.52	0.01
		1000	5869.7			

Table 5. Results for robustness study (no. of determinations=2) of ofloxacin

S. No.	Parameter checked	Conc. ng	Area	Mean	SD	%RSD
1.	**Increased saturation time by 30 minutes**		**OFLO**	**OFLO**	**OFLO**	**OFLO**
		1000	5235.4			
		1000	5355.3	5285.1	0.63	0.71
		1000	5264.7			
2.	**Increased migration distance by 80 mm.**	1000	5548.4			
		1000	5382.4	5467.0	1.34	0.01
		1000	5470.3			

Table 6. Results of Ruggedness study of CEF

Analyst -1

Conc.	Area	Mean	S.D.	%R.S.D.
1000	5829.6			
1000	5456.8	5469.9	0.14	0.145
1000	5123.3			

Table 7. Results of Ruggedness study of OFLO

Analyst-1

Conc.	Area	Mean	S.D.	%R.S.D.
1000	5736.8			
1000	5802.1	5843.4	0.07	0.072
1000	5991.4			

Table 8. Results of Ruggedness study of CEF **Analyst -2**

Conc.	Area	Mean	S.D.	%R.S.D.
1000	5879.6			
1000	5378.7	5653.5	0.28	0.278
1000	5702.4			

Table 9. Results of Ruggedness study of OFLO **Analyst-2**

Conc.	Area	Mean	S.D.	%R.S.D.
1000	5034.9			
1000	5366.7	5421.5	0.07	0.079
1000	5862.9			

Table 10. Results of Specificity study of CEF

Addition	Conc.	Peak Area	% amount found	%RSD
Talc	1500	8675.4	99.6	0.431
Starch	1500	9804.7	100.4	0.140
MCC	1500	6754.3	101.7	0.069

Table 11. Results of specificity study of OFLO

Addition	Conc.	Peak Area	% amount found	%RSD
Talc	1500	7562.9	100.5	0.072
Starch	1500	8275.3	101.8	0.139
MCC	1500	6688.3	99.3	0.213

bulk and combined tablet dosage form. In this proposed method the linearity is observed in the concentration range of 500-3000ng/spot for both CEF and OFLO with coefficient of correlation, (r^2) =0.9979 and (r^2) =0.9971for CEF and OFLO, respectively at 255 nm. The result of the analysis of pharmaceutical formulation by the proposed method is highly reproducible and reliable. Method appear good agreement with the label claim of the drug. The method can be used for the routine analysis of the CEF and OFLO in combined dosage form without any interference of excipients.

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Delivery of Recombinant Plasmid Containing Human Insulin Gene-GLP1 Promoter into L Cells in the Rats with Type-1 Diabetes

Sima Ataollahi Eshkoor[a] and Zalinah Ahmad[b]
[a]Iranian Research Center on Aging, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran
[b]Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
*For Correspondence - simaataolahi@yahoo.com

Abstract

The purpose of this study was to determine whether the treatment with recombinant plasmid consisted of human GLP1 promoter and insulin gene can treat diabetic rats. Rats were induced type-1 diabetes mellitus (T1DM) by a single dose of intraperitoneal injection of streptozotocin (STZ) at dose of 55mg/kg. The induction of diabetes was confirmed in rats by checking the blood glucose level for seven days. The recombinant plasmid, GLP1/Ins/pBud plasmid, was wrapped with chitosan and then transferred to diabetic rats by force feeding. The blood glucose level was checked from the tips of the tails by needle puncture using a glucometer and test strips. The blood levels of human and rat insulin were assessed by enzyme-linked immunosorbent assay (ELISA). The results showed no significant effects of orally treatment with recombinant plasmid DNA at both doses of 100 and 600 µg/mL on the human insulin level in diabetic rats ($p>0.05$). The human insulin level was significantly increased by orally treatment at dose of 300 µg/mL ($p=0.04$). The findings indicated that the intraperitoneal injection of 300 µg/mL of this nanoparticle complex prominently increased the human insulin level in diabetic rats in contrast to both doses of 100 and 600 µg/mL. Despite above results, both methods was not effective enough to decrease the blood glucose levels in diabetic rats. It was concluded that the treatment of diabetic rats with recombinant plasmid consisted of human GLP1 promoter and insulin gene was not effective to reduce the blood glucose levels in diabetic rats.

Keywords: Diabetes Mellitus; T1DM; Plasmid; Chitosan; L cells

Introduction

Type-1 diabetes (T1DM) is a chronic autoimmune disease caused by damaging the beta-cells in the islets of Langerhans. This destruction results in insulin deficiency and hyperglycemia (1). Poor glycemic control in diabetes can cause severe secondary complications. Exogenous insulin cannot completely prevent complications of diabetes, thereby morbidity and mortality of diabetes are increased significantly (2). In 2007, 437,500 children were affected by type 1 diabetes worldwide. Approximately, 70,000 children less than 14 years old develop T1DM per year with an annual increasing rate of 3%. The primary clinical signs of T1DM are ketoacidosis and chronic hyperglycaemia. Diabetic ketoacidosis can lead to coma and death. In addition, chronic hyperglycaemia can cause macrovascular and microvascular complications such as retinopathy, peripheral neuropathy, cardiovascular disease, and renal disease (3). It is a plan to treat type-1 diabetes by gene therapy through in vivo or in vitro transfer of insulin gene into germ line or somatic cells (4). One of target cells in gene therapy is gasterointestinal L-cells, which produce Glucagon-like peptide 1 (GLP-1)

hormone (5). This hormone is an incretin hormone that is able to promote insulin secretion, inhibit the glucagon secretion in pancreas, and increase the synthesis of proinsulin. Furthermore, GLP-1 promotes the proliferation of β-cells, and inhibits β-cell apoptosis. Meanwhile, it enhances the hepatic glucose uptake following increased glycogen syntheses activity (3).

A specific part of proglucagon promoter can activate L cells to produce GLP-1 and insulin (6). In addition, the engineered L-cells can be used to synthesise, process and secrete mature insulin (7). Thus, the combination of GLP-1 promoter with insulin gene transferred by nanoparticles in the gastrointestinal cells can potentially regulate the blood glucose level and treat T1DM (5). The human insulin gene can be transferred by chitosan-DNA nanoparticles in the gastrointestinal tract (8,12). Chitosan is a biodegradable polysaccharide composed of D-glucosamine and N-acetyl-glucosamine. It is a safe, nontoxic and cationic carrier (9) that can be used as a carrier to deliver GLP-1 gene and transfer insulin gene into the gastrointestinal epithelial cells of L cells (10) in order to treat diabetes. However, the exact cause of T1DM is unknown. Apparently, a genetic predisposition, environmental factors and distinctive metabolic changes contribute to initiation, development and progression of diabetes (3). There are many attempts to cure diabetes. Thus, this study aimed to investigate the effects of gene therapy via transferring plasmid-insulin gene-GLP1 promoter into the gastrointestinal L cells on the regulation of glucose and insulin levels in the blood of diabetic rats.

Methods

DNA extraction: Bacteria (*E.coli* strain TOP-10F) in frozen glycerol stocks were grown on LB agar (Lysogeny broth) (1st Base, Malaysia) plates without antibiotics overnight. A single isolated colony from the plate was cultured into 5 ml of LB broth medium (1st Base, Malaysia) (without antibiotics) at 37°C with shaking overnight. After that, the harvested cells were centrifuged at 9000 rpm for 30s to collect bacterial pellet. The extraction procedure was continued by following the protocol of High-purity Plasmid DNA Mini-Preparation Kit (BioTeke Corporation; China). The extracted DNA concentration was measured by nanometer drop (Eppendorf AG, Hamburg, Germany).

Preparation of Nanopaticle: The preparation of DNA-chitosan nanoparticles was based on a protocol as previously described (11). Briefly, chitosan was dissolved in sodium acetate followed by mild stirring and heating at about 60°C overnight to form chitosan solution. Equal volumes of filtered 0.02% chitosan in 5mM sodium acetate buffer and 100 μg/mL plasmid-DNA in 50mM sodium sulfate were preheated to 55°C, then mixed together quickly and vortexed for 30s. This mixture was kept at room temperature for 30 min that was for stabilization. The chitosan powder with the medium molecular weight and 75-85% deacetylated (Sigma, USA) was used to prepare chitosan-DNA nanoparticles. Meanwhile, particle size and charge characterizations were carried out using a Zetasizer (Nano ZS-Malvern) at 25°C. This optimization was through various concentrations of chitosan and different pH levels of sodium acetate solution. In addition, the transmission electron microscopy (TEM) (Hitachi H-7100 TEM) was used to evaluate the distribution and morphology of nanoparticles. A DNase degradation test was applied to assess the efficiency of encapsulation and DNA stability against nuclease degradation.

Procedure: The effect of human insulin gene transferring was studied in diabetic rats using GLP1/Ins/pBud plasmid. Plasmid consisted of the human insulin gene and the specific promoter of L-cells. This recombinant plasmid delivered and transferred into animal model using chitosan nanoparticles as gene carrier. Rats were divided into three sub-groups, where each subgroup contained 12 rats. The first diabetic group received nanoparticles containing GLP1/Ins/pBud plasmid. The second group was diabetic control and only treated with chitosan. The last group was healthy rats that did not receive STZ

injection or treatments. Nanoparticles were administered intraperitoneally and orally to diabetic rats. The treatment doses were 100, 300 and 600 µg/mL of DNA solution in a 300 µL volume for each rat. The repetition for oral and intraperitoneal treatment was 7 and 4 times respectively that was repeated every two days.

Determination of insulin efficiency through blood glucose testing: The effects of recombinant plasmid consisting human insulin gene on the blood glucose level in diabetic rats were studied. Following the nanoparticle treatment, the blood glucose concentration was measured every other day for 35 days in all groups. The blood glucose level was checked from the tips of the tails by needle puncture using a glucometer and test strips (Expeed VIVO, HuBDIC, Korea). The blood glucose levels in the treated groups were compared with the untreated diabetic rats and healthy rats.

Detection of insulin protein by ELIZA: The human insulin protein secretion in blood was detected using the ultrasensitive human insulin ELIZA kit (ALPCO, USA). The serum was separated from blood and used for the ELIZA test. Blood was collected from rats' heart, incubated at 37°C for 1 hr and allowed to clot. Then, blood samples were centrifuged at 3000 rpm for 10 min. The yellowish serum in the supernatant was transferred to a new tube and stored at -80°C.

ELIZA Test for Human and Rat Insulin: The amount of insulin present in the serum collected from blood samples was determined using the ultrasensitive human and rat insulin ELIZA kits (ALPCO, USA). The human ELIZA kit had 100% cross reactivity with mature human insulin, whereas no reaction was reported with other forms, such as pro-insulin, C-peptide. ELIZA test was carried on as below. An about 25 µl of each standard (reconstituted control) and sample were loaded into their respective wells. Then 100 µl of detection antibody was added to each well. The microplate was covered and incubated for 1 hr at room temperate on a shaker (700-900 rpm). After that, the microplate was washed six times with working strength wash buffer. After the final wash step, any residual wash buffer and bubbles was removed from the wells by inverting and firmly tapping the microplate on absorbent paper towels. After that, 100 µl of TMB substrate was added to each well of microplate and then was covered and incubated for 30 min at room temperature on a shaker (700-900 rpm). The stop of reaction was achieved by adding 100 µl of stop solution to each well and then shaking gently. Finally, the microplate was analysed using a Bio-Tek EL800 Microplate Reader (LABEQUIP, Canada) with 450 nm filter. In the rat insulin ELIZA kit, the 96-well microplate was coated with a monoclonal antibody that was specific for insulin. About 10 µl of standards, controls, and samples were added into the microplate wells and then mixed with 75 µl of Working Strength Conjugate. The microplate was sealed and incubated at room temperature on a microplate shaker at 700-900 rpm. After that, wells were washed 6 times with wash buffer and then well dried. About 100 µl of TMB substrate was added into each well and then the microplate was covered and incubated for 15 min at room temperature on a shaker (700-900 rpm). Once incubation finished, 100 µl of stop solution was added, and then the optical density (OD) was measured by a Bio-Tek EL800 Microplate Reader (LABEQUIP, Canada) at 450 nm. The calculation of insulin level for both human and rat insulin kits was based on manufacture recommends.

Induction of T1DM in Rats: Animal study was on albino rats according to the guidelines for the care and use of experimental animals and the ethics committee that was approved by the Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM). Type I diabetes mellitus was induced in albino rat model, 7-8 weeks old using streptozotocin (STZ; N-nitroso derivative of glucosamine) (Sigma, USA). Rats were starved overnight and then treated with a single intraperitoneal injection of 55mg/kg (mg per body weight) STZ. STZ was dissolved in cold

Na-Citrate solution (0.01 M citrate buffer, pH 4.5) and injected within 5 min to avoid degradation of STZ. After that, rats were supplied with 10% sucrose water overnight to avoid sudden hypoglycemia post-injection. Animals had access to food and water. When the blood glucose level was increased greater than 27.75 mmol/L for 7 consecutive days (12), the injected rats were considered as T1DM cases (13) for subsequent experiments. Animals had access to food and water. Readings were usually taken in morning when rats were under fasting and no fasting conditions. Glucometer read “HI” if the blood glucose exceeded 33.3 mmol/L. Those readings were set on 33.3 mmol/L for averaging.

Results and Discussion

The results showed that the orally treatment of albino rats with GLP1/Ins/pBud plasmid was not significantly effective to decrease the blood glucose level ($p>0.05$). Giving orally different doses of recombinant plasmid including 100, 300 and 600 µg/mL to diabetic rats did not significantly decrease the fasting and no fasting blood glucose levels in diabetic rats ($p>0.05$). After the orally treatment of diabetic rats with 300 µg/mL of DNA solution, the release of human insulin and rat insulin was significant ($p= 0.04$) and non-significant ($p> 0.05$), respectively. Tables 1 and 2 show the mean values of blood levels of glucose and insulin after the treatment of diabetic rats with GLP1/Ins/pBud plasmid. The injection of 100, 300 and 600 µg/mL doses of DNA solution in few diabetic rats showed that a dose concentration of 300 µg/mL prominently increased the level of human insulin secretion compared to the concentrations of 100 and 600 µg/mL (Table 1). Such effects are more likely due to the multiple mechanisms involving in the regulation of insulin gene expression attributed to nutrients, hormones and pharmacological factors (14, 15).

The human insulin level after treating with the concentration of DNA solution at 600 µg/mL was less than 300 µg/mL that was probably due to hyperinsulinemic-insulin resistance following the impaired L cells and GLP-1 release in response to insulin and heterologous GLP-1 secretagogues (16). Contrary to the report of Bowman and co-workers (17), our study showed that the increased dose of DNA solution to 300 µg/mL elevated the efficiency of gene delivery regarding the enhancement of insulin level. It was probably due to the increased plasma copy numbers and transferred insulin gene to L cells. However, the results showed that T1DM was not reversal to normal state after this treatment. Our project showed a relative impact of transferred human insulin gene and GLP1 promoter into the gastrointestinal L cells carried by nanoparticle chitosan for releasing insulin in the diabetic cases. Such effect has been previously reported by Jean and colleagues (10) indicating such function of gastrointestinal epithelial cells and GLP1 on the increased level of insulin. Until now, exogenous insulin or gene transfer for insulin or glucokinase alone has failed to treat diabetes. Thus, further investigations are required to clarify the synergistic action of insulin and glucagon promoter to treat diabetes. Accordingly, this study aimed to investigate the effects of the transfer of the insulin gene and GLP1 promoter on the regulation of blood insulin and glucose levels in animal model for treating diabetes.

This study confirmed previous reports those introduced plasmid as a non-viral carrier of DNA in gene therapy conducted by chitosan (8, 10, 18, 19). Contrary to some earlier research (8, 19), our results showed non effectiveness of the transfer of the human insulin gene in rats to decrease blood glucose level. Such effect can be related to genetic polymorphism in rats (1), and the type of insulin in each species. Human insulin is similar to bovine and porcine insulins. Porcine and bovine insulins structurally differ from human insulin in one amino acid (alanine replaces threonine at B30) and three amino acids (A8 alanine, A10 valine and B30 alanine), respectively. Bovine insulin is more immunogenic than both porcine and human insulins (20).

In addition, rapid turnover of intestinal epithelial cells can decrease the efficiency of transfection of recombinant plasmid DNA in the

gastrointestinal L cells (21). These cells are in the distal jejunum, ileum, colon, and rectum (22). The maximum density of L-cells is in the ileum in most species (23). While intestinal epithelial cells renewal in mammals is 3-5 days (21), average ileum turnover in rats is 1.4 days (24).

The results can also be associated with the type of chitosan, which can affect gene delivery and DNA chitosan nanoparticles. Such effect can be related to the variety of chitosan in molecular weight and degree of deacetylation. Since, lower degree of deacetylation of chitosan can reduce the efficiency of DNA binding and stability of nanoparticle complex in a dose dependent manner (17); therefore, further optimization is needed to find a synthetic degradable cationic polymer for protecting plasmid effectively and release. However, the results were against what we expected. Such insufficiency may reflect slow intracellular DNA release from chitosan nanoparticles (17). The findings of our in vivo study did not confirm the results of gene delivery found in an in vitro research by Rasouli and co-workers (5). It suggests that the results achieved by in vivo studies may not always be similar to in vitro results. This difference is a confirmation to the presence of disparity amongst different studies (17), which can be due to diversity in genetic polymorphism and immune responses (1).

All research showed no therapeutic effects of treatment with gene transfer on diabetes, which can be attributed to low levels of insulin and high variability in gene delivery (17). However, the technique of gene delivery using nanoparticles tries to produce detectable amount of insulin in order to treat diabetes. Accordingly, this study aimed to use plasmid as a non-viral gene carrier to deliver human insulin gene and GLP1 promoter to diabetic rats following in vitro study by Rasouli and colleagues for treating T1DM (5).

In the current study, survival rate in sample groups was significantly more than diabetic control groups ($p<0.001$). Mortality rate in diabetic rats after treatment with recombinant plasmid was less than those without treatment. The period time of survival in diabetic rats was more than 60 days without having any objective problems such as wound, which was more than expectation in severe diabetes with a blood glucose level more than 33.3 mmol/L. Such effect was possibly related to the reduction of complications after treatment. For example, positive impact of this treatment on GLP-1 receptors may subsequently prevent complications such as cardiovascular diseases and neuronal degeneration (9). The subsequent stimulation of PI-3K signaling pathway (25) and MAP Kinase signaling pathway (26) can aslo result in cell growth and lifespan extension. However, some problems including DNA-plasma degradation in GI tract, partial degradation of plasmid DNA during the transport of chitosan nanoparticles complex across cells, incomplete DNA protection (27) and the poor intracellular release of DNA from chitosan nanoparticle complexes (28) can limit utility of oral gene therapy as a method to treat diabetes completely. Such limits can decrease the expression level of insulin gene in the cells (17). Furthermore, the expression of insulin gene following gene therapy and using nanoparticles is valid for a limited period and needs to be repeated periodically after shedding epithelial cells. Meanwhile, lack of typical prohormone convertase for processing of proinsulin to insulin hormone in gastrointestinal cells such as L cells can lead to partially production of insulin, which limits use of these cells as target cells in gene therapy for diabetes (8). Our study was the first research, which used plasmid with both insulin gene and GLP1-promoter wrapped with chitosan for treating T1DM in animal model. Further investigations are needed on stem cells, gene polymorphism, immune reactions and system controls for the proliferation and regeneration of gastrointestinal cells such as L cells and K cells for finding a better approach to treat diabetes. Meanwhile, the increase of therapeutic effects of gene delivery by nanoparticles requires further studies regarding gene transfer barriers, exact cells for transfection, proper stage for releasing

DNA from chitosan and optimal optimization for polymer carrier.

Limitations : Despite challenges, the strategy of gene delivery into the gastrointesinal cells using the complex of plasmid containing insulin gene and nanoparticle regarding the treatment of diabetes needs further examination. The success of treatment is associated with different parameters such as the efficiency of gene transfer via oral administration. Our project have been faced some limitations, which could affect the interpretation of findings. Biological differences between human and rat was a limit that might cause different reactions to insulin and glucose levels. In addition, our study was limited by the modest levels of produced insulin in the intestinal cells and high variability in gene transfer through gene therapy by nanoparticles. A large numbers of L cells transfected with insulin gene are needed to produce enough insulin for the treatment of diabetes. Meanwhile, the long term action of insulin may have a limitation. However, the shedding of gastrointestinal epithelial cells such as L cells can decrease their efficiency as target cells in gene therapy. The release of transgenic DNA from chitosan-DNA nanoparticles in the bloodstream and transport to systemic tissue by crossing the gastrointestinal tract can affect the efficient transport of DNA to the gastrointestinal cells such as L cells as well.

Conclusions

Our results suggest that the transport of transfected insulin gene into L cells could secrete human insulin hormone and execute relative effects on diabetic rats in a dose dependent manner. It was concluded that the concentration of 300 µg/mL of DNA solution was the efficient dose compared to doses including 100 and 600 µg/mL to increase the level of human insulin protein in diabetic rats. The blood glucose level

Table 1. Insulin levels after treatment with recombinant plasmid DNA (100, 300,600 µg/mL) in diabetic rats

Human Insulin Level				Rat Insulin Level		
Conc. (µg/mL)	100	300	600	100	300	600
Orally	0.83±0.72	1.32±0.88	0.36±0.20	0.06±0.03	0.09±0.07	0.10±0.04
Injection	0.6±0.78	4.16±0.02	0.21±0.16	0.06±0.02	0.07±0.00	0.09±0.04

Conc.= Concentration dose

Table 2. FBS and NFBS Glucose levels after the treatment with recombinant plasmid DNA (100, 300,600 µg/mL) in diabetic rats

FBS Glucose level (mmol/L)				p value	NFBS Glucose level (mmol/L)			p value
Conc. (µg/mL)	100	300	600		100	300	600	
Orally	22.8±6.89	30.5±5.40	22.4±5.73	0.12	32.6±1.54	31.5±1.59	33.3±0.00	0.39
Injection	32.8±0.75	33.3±0.00	28.3±3.69	-	29.7±5.15	33.3±0.00	26.7±6.96	-

*p less the 0.05 is significant using one way-ANOVA analysis test
FBS=Fasting blood glucose; NFBS=No fasting blood glucose
Conc.= Concentration dose

was not significantly decreased after the treatment with all concentrations. The findings indicated that the transport of GLP1/Ins/pBud plasmid into L cells wrapped with chitosan stimulated the release of insulin hormone into the bloodstream in a dose dependent manner. This study supports the view that the transport of transfected insulin gene into L cells can be a potential approach to regulate insulin level, make free patients from repeated insulin injections and reduce complications. However, further investigations are needed to prove the efficiency of gene therapy and genetic engineering using L cells in the treatment of diabetes mellitus.

Acknowledgments

Authors would like to acknowledge Mina Rasouli for donating provided bacteria having plasma-DNA-GLP1 combination from her own study.

Conflict of interest: Authors declare that there is no conflict of interests

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Vol. 10 (1) 72-77 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Potential of Surface Sterilants to Control the Microbial Contamination of field grown Fir (Abiespindrow Royle.) Seedling Explants Intended for in vitro Culture

Bhat, S. J. A.[1*], Syed Maqbool Geelani[2], S. A. Gangoo[1] and H. A. Bhat[1]
[1]Faculty of Forestry,Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Benihama-Watlar, Ganderbal- 191 201 (J&K).
[2]Division of Environmental Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar -191 121
*For Correspondence - javaidforest11@gmail.com

Abstract

An effective disinfection protocol for micropropagation of fir (*Abies pindrow*Royle.) using nodal and internodal segments as explants was developed. The explants were sterilized with different sterilants for different time duration. The present studies on the effect of different sterilization regimes revealed that maximum aseptic cultures were obtained from both explants nodal and internodal segments when treated with combination of 0.1% mercuric chloride for 7 minutes and 10% sodium hypochlorite for 15 minutes, but surviving percentage was low because this treatment resulted in necrosis and tissue injury of explants. However, mercuric chloride (0.1%) for 10 min resulted in low percentage of aseptic culture but gave highest percentage of surviving explants as most of researchers found that the single sterilant is more effective than the combination.

Key words: Sterilization, contamination, *Abies pindrow*, *in vitro*

Introduction

Microbial contaminations present a major challenge to the initiation and maintenance of viable *in vitro* cultures. These contaminants are particularly dangerous when they are plant pathogens. The problem is further exacerbated when explants material is sourced directly from field grown plants. Contamination in this paper refers to fungi or bacteria naturally present on the surface and natural openings on the explants material, which become manifested after initiation and can either, be overt or covert. Overt refers to contamination that can be identified by visible inspection, whereas covert refers to latent contamination, which requires special indexing and/or assaying techniques for identification. The fir (*Abies pindrow*Royle), a member of Pinaceae family has significance throughout the Western Himalayas from Afghanistan to Nepal due to their demand as wood-based raw material for shook industry, packing cases, match wood, medicine, ornamental, newsprint etc. Fir is preferred for use as timber for beams, rafters, purlines, trusses and framework (1). These qualities increases the economic importance of this species throughout the region.

Abies pindrow : Royle is generally propagated through seeds, Germination and survival under natural condition is poor and highly variable. This is due to various factors like low soil moisture retentivity, excessive grazing, thick layer of humus, dense weed growth, small seed size, poor seed production, infrequent seed years and slow decomposition rate of litter fall (2). Conventional vegetative propagation of *Abiespindrow*Royle.captures all the characters of mother plant, but multiplication rate is too slow,

and there is potential for virus transfer using cuttings.

Tissue culture techniques allow rapid multiplication of plantlets obtained from different explants through direct or indirect morphogenesis. The division of offshoots are not always suitable for this type of propagation due to their vulnerability and susceptibility to pathological agents. Several studies have attested the tissue cultured plants being more advantageous than those by conventional propagation in terms of problems related to improvement especially in forest trees (3). Micropropagation of conifers from different explants has been reported and may be applied to regenerate disease free plants (4 and 5). The Scot pine (*Pinus sylvestris* L.) propagated from vegetative samples are often infected due to seasonal effects in the explant viability and degree of contamination (6). If we can obtain healthy explant materials from offshoot, this problem will be overcome. But the offshoot samples larger than explant size makes disinfection difficult. Contamination leading to browning in the initial establishing stage of *in vitro* culture is the main cause leading to explant death (7). According to (8), explants of *Abies spps.* surface sterilized with 0.1 to 0.2% mercuric chloride (HgCl2) for 10-15 minutes gave satisfactory contamination control. However, there is no published treatment efficacy data on *Abies pindrow* Royle. In the present study, effect of mercuric chloride (0.1%) and sodium hypochlorite (10%) alone and in combination for varying contact time duration was studied on explant disinfection. This study identifies problems of microbial contamination in *in vitro* cultures, and attempts to develop a protocol for disinfecting the field grown explants intended for *in vitro* culture. The objective was development of an efficient and simple disinfection protocol to increase survival of *A. pindrow* explants.

Materials and Methods

Chemical control of microbial contamination of two explants (nodal and internodal segments) of fir (*Abies pindrow* Royle) under *in vitro* conditions was conducted. The experiment analysed the efficiency of disinfecting agents and time of their operation on the percentage of explants of fir (*Abies pindrow* Royle) not infected by bacteria or fungi. Two disinfecting agents were tested *viz*. mercuric chloride (HgCl2) and sodium hypochlorite (NaOCl) on both explants. The type, concentration, combination of the disinfecting agents and the contact time on the explants were adjusted (Table-1).

Before disinfection, the explants were washed with Tween 20 detergent for 5 min then rinsed with water for 5-6 times. After washing, the explants were reduced in size by removing tissues of size (0.5-1.0 cm) with the help of surgical blade and forceps before inoculation. After washing the explants, they were brought to laminar flow cabinet and rinse with sterile distilled water under aseptic conditions. Treated explants were placed on growth medium in such a manner that conformed to the original polarity. MS basal medium (9) was used throughout the study. Appropriate quantities of various stock solutions and plant growth regulators were pipetted out and stirred with 400 ml distilled water. After adding sucrose at a concentration of 3%, pH was adjusted to 5.7 with 0.1 N NaOH and 0.1 HCl. Agar media at concentration of 0.7% was added and the final volume was made to 1 L with distilled water. The medium was sterilized in an autoclave at 15 psi (121°C for 15 min). The interior surface of laminar flow cabinet was first swabbed with 95% ethanol and all the required materials except living plant tissues were kept inside the chamber and exposed to UV light for 60 min. The laminar flow was switched on 10 min prior to inoculation or sub culturing. The culture room used for incubating the culture was maintained at temperature of 24±1°C by regulating the room air conditioner or thermostatically controlled heater as per requirement. For light, flourescent light tubes of 3000-3200 lux were fixed to maintain 16 h photoperiod.

Observations on percentages (%) of aseptic cultures, necrotic cultures and explant survival were made within 15 days of inoculation. Each treatment combination was assigned to 10 explants with one explant per test tube and replicated five times. The data generated was subjected to ANOVA in complete randomized design using R- software at 5% level of significance. To satisfy model, assumptions of experiments were subjected to arc sine and square root transformations. The significant difference among treatments was compared by critical difference.

Results and Discussion

Fir explant, nodal and internodal segments, were subjected to eight different sterilization regimes and viability assessed using (Murashige and Skoog, 1962) (MS) as the basal medium. Effect of various sterilization regimes and explants on culture asepsis, necrosis and explant survival (Table-2) was highly significant. Best aseptic cultures (58.8%) were obtained by treating the explants with 0.1% mercuric chloride for 7 minutes and 10% sodium hypochlorite for 15 minutes. The aseptic frequency of nodal segment explants was significantly higher (61.5%) than the internodal segments explants (34.4%). Interaction studies showed that a maximum culture asepsis of (75.3%) when the nodal segments were treated with 0.1% mercuric chloride for 7 minutes and 10% sodium hypochlorite for 15 minutes.

The highest necrotic cultures (38.1%) were obtained when explants were surface sterilized with 10% sodium hypochlorite for 20 minutes. The lowest mean (21.6%) of necrotic cultures was obtained by treating the explants with mercuric chloride 0.1% for 10 minutes sterilization regime. The per cent response of necrotic cultures of nodal segment explants was higher (32.0%) than the internodal segments (30.0%). Interaction studies show maximum percentage of necrotic cultures (51.0%) when the nodal segments were treated with 0.1% mercuric chloride for 5 minutes and 10% sodium hypochlorite for 10 minutes and (43.3%) when internodal segments were treated with mercuric chloride ($HgCl_2$) for 5 minutes and lowest necrotic cultures (13.3%) was obtained when explants were surface sterilized with mercuric chloride 0.1 per cent for 10 minutes.

The highest percentage of surviving cultures (45.0%) was obtained by treating the explants with mercuric chloride (0.1%) for 10 minutes. The percent treating response of internodal segment explants was significantly higher (33.9%) than the nodal segments explants (30.2%). The lowest mean survival of (23.3%) was obtained when the explants were treated with 10% sodium hypochlorite for 20 minutes.

Table 1. Concentration of various sterilants and time duration for surface sterilization of different explants

S. No.	Sterilant	Time
1	Mercuric chloride ($HgCl_2$) (0.1%)	5 min
2	Mercuric chloride ($HgCl_2$) (0.1%)	7 min
3	Mercuric chloride ($HgCl_2$) (0.1%)	10 min
4	Sodium hypochlorite (NaOCl) (10%)	10 min
5	Sodium hypochlorite (NaOCl) (10%)	15 min
6	Sodium hypochlorite (NaOCl) (10%)	20 min
7	Mercuric chloride (0.1%) and Sodium hypochlorite (10%)	5 min and 10 min
8	Mercuric chloride (0.1%) and Sodium hypochlorite (10%)	7 min and 15 min

Table 2. Effect of different sterilization regimes on per cent aseptic cultures, explant survival and necrotic culture in fir (Abies pindrow Royle.)

Sterilants (time duration)	**Aseptic culture (%)			*Explant survival (%)			*Necrotic culture (%)		
	Nodal segments	**Internodal segments**	**Mean**	**Nodal segments**	**Internodal segments**	**Mean**	**Nodal segments**	**Internodal segments**	**Mean**
Mercuric chloride (0.1%) (5 min)	46.00 (43.06)	30.00 (33.19)	**38.33 (38.13)**	30.00 (5.56)	26.66 (5.23)	**28.33 (5.40)**	20.00 (4.57)	43.33 (6.64)	**31.66 (5.61)**
Mercuric chloride (0.1%) (7 min)	49.00 (44.41)	33.33 (35.20)	**41.16 (39.80)**	31.33 (5.68)	43.33 (6.64)	**37.33 (6.16)**	16.33 (4.16)	36.66 (6.12)	**26.50 (5.14)**
Mercuric chloride (0.1%) (10 min)	56.66 (48.83)	36.66 (37.21)	**46.66 (43.02)**	43.33 (6.64)	46.66 (6.87)	**45.00 (6.76)**	13.33 (3.68)	30.00 (5.56)	**21.66 (4.62)**
Sodium hypochlorite (10%) (10 min)	60.00 (50.82)	23.33 (28.76)	**41.66 (39.79)**	30.00 (5.56)	30.00 (5.51)	**30.00 (5.53)**	30.66 (5.62)	26.66 (5.23)	**28.66 (5.43)**
Sodium hypochlorite (10%) (15 min)	63.66 (52.93)	28.33 (32.12)	**46.00 (42.53)**	26.66 (5.24)	30.00 (5.51)	**28.33 (5.38)**	36.66 (6.12)	28.33 (5.38)	**32.50 (5.75)**
Sodium hypochlorite (10%) (20 min)	68.00 (55.57)	43.33 (41.13)	**55.66 (48.35)**	23.33 (4.90)	33.33 (5.84)	**28.33 (5.37)**	46.33 (6.87)	30.00 (5.51)	**38.16 (6.19)**
Mercuric chloride (0.1%) and Sodium hypochlorite (10%) (5 min + 10 min)	72.66 (58.49)	38.33 (38.18)	**55.50 (48.34)**	27.00 (5.28)	29.33 (5.49)	**28.16 (5.39)**	51.00 (7.20)	20.66 (4.64)	**35.83 (5.92)**
Mercuric chloride (0.1%) and Sodium hypochlorite (10%) (7 min + 15 min)	75.33 (60.22)	42.33 (40.54)	**58.83 (50.38)**	30.00 (5.56)	32.00 (5.73)	**31.00 (5.64)**	48.66 (7.04)	24.33 (5.02)	**36.50 (6.03)**
Mean		**61.50 (51.79)**	**34.45 (35.79)**		**30.20 (5.55)**	**33.91 (5.85)**		**32.00 (5.66)**	**30.00 (5.52)**

Values in the parenthesis are **arc sine and *square root transformed.

Interaction studies showed that the maximum percentage of surviving explants was (46.6%) when the internodal segments were treated with mercuric chloride (0.1%) for 10 minutes.

Plant tissues carry wide range of microbial contaminants. Surface contaminants can significantly reduce the success of micropropagation operations, especially in less well studied species such as *A. pindrow*. To avoid these sources of contamination in tissue culture operations, plant tissues must first be surface sterilized before planting it on nutrient medium. The present studies on the effect of different regimes of sterilization revealed that maximum aseptic cultures were obtained from both explants (nodal and internodal segments) when treated with 0.1% mercuric chloride (HgCl2) for 7 minutes and 10% sodium hypochlorite (NaOCl) for 15 minutes, but the surviving percentage was low because this treatment resulted in necrosis and tissue injury of explants. However, mercuric chloride (0.1%) for 10 minutes resulted in less percentage of aseptic cultures but gave highest percentage of surviving explants. The similar findings have already been confirmed by many workers that a single sterilant is more effective than the combination. These results are in close conformity with those of (10) in *Abies alba* and (11) in *Abies concolor × Abies grandis*. Likewise, (12) showed that application of mercuric chloride in a concentration of 0.1-0.2% applied for 10-15 minutes gave effective surface sterilization procedure for maximum survival of explants with minimum injury on micro-propagation of *Abiesalba × Abiesnordmanniana.* (13) reported that conifer bud explants treated with 10% sodium hypochlorite for 10 minutes proved most quite efficient agent.

Conclusion

Successful application of plant tissue sterilants increases the likelihood of healthy micro-propagated seedlings. In this study different plant tissue surface decontamination treatments applied for different contact durations yielded aseptic cultures using *A. pindrow* explant tissue. The highest percentage of aseptic culture was achieved by treating the explants with 0.1% mercuric chloride for 7 min and 10% sodium hypochlorite for 15 min. The highest percentage of explant survival was obtained when explants were sterilized with mercuric chloride (0.1%) for 10 min. The use of 0.1% mercuric chloride for 7 min and 10% sodium hypochlorite for 15 min gave the highest aseptic cultures but resulted in higher necrotic cultures. So it is concluded from the above study that sterilization treatment of mercuric chloride (0.1%) for 10 min is effective for disinfecting the field grown explants of *Abies pindrow* intended for *invitro* culture. The treatment resulted in maximum percentage of explant survival.

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Vol. 10 (1) 78-91 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Applications of Nanotechnology in Drug Delivery and Design - An Insight

Janith Wanigasekara and Chamindri Witharana*
Department of Biochemistry and Molecular Biology, Faculty of Medicine,
University of Colombo, Sri Lanka
*For Correspondence- chamindri@bmb.cmb.ac.lk

Abstract

Nanotechnological application is greatly important in the field of drug delivery because of its high specificity towards the target site, so it is able to reduce toxic side effects of drugs to normal cells. Reduce plasma fluctuation of drugs, high solubility, efficiency, reduces cost of products and enhancement of patience comfort are reasons that nanotechnology is used for drug delivery. The nanoparticle (NP) plays a vital role and it can conjugate with various drugs by different methods to deliver drugs to the target site. The NP surface is designed with ligands to get affinity towards specific cells and co-polymers to get protection from immune cells. The nanoparticles conjugated drug can eventually recognize the site and join to the target and enter to the cell by receptor mediated endocytosis. Then NPs are able to release drugs controllably to cure diseases. This review analyses the nanotechnology in drug delivery, discovery, nanoparticles and formulation, mechanism of drug delivery and applications.

Keywords: Nanoparticles, Nanoparticle formulation, history, Drug delivery system, mechanism of delivery.

Abbreviations

Acquired immunodeficiency syndrome (AIDS), Atomic force microscope (AFM), Blood brain barrier (BBB), Carbon nano-horns (CNH), Carbon nano-tubes (CNT'S), Drug delivery system (DDS), Elastomer-like proteins (ELP), Genetic engineering method (GEM), Human immunodeficiency virus (HIV), Inosiazid (INH), Lipid drug conjugates (LDC), Metal nano-particles (MNP), Multiple drug resistance (MDR), Nano structured lipid carriers (NLC), Nanoparticles (NP), National aeronautics and space administration (NASA), Polyethylene glycol (PEG), Poly-glycolic acid (PGA), Poly-lactic acid (PLA), Pyrazinamide (PZA), Rafampin (RMP), Screening tunneling microscope (STM), Solid lipid nano-particles (SLN), Tuberculosis (TB).

Introduction

National nanotechnology initiatives in USA, defined nanotechnology as "Science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers". The study, design, synthesis, manipulation, and application of functional materials at nanometer scale and one nanometer being equal to 1×10^{-9} m that is at the atomic and molecular levels (1).

Nanotechnology is recently developed science and it is able to create engineering functional materials or systems, devices with in the nanoscale. Nanomaterials have unique properties such as mechanical, optical, magnetic, electrical and biochemical with vast range of applications ranging from basic material science to personal care applications. Some recently developed applications of nanotechnology are energy storage production and conversion, agriculture productivity enhancement, water treatment and remediation, disease diagnosis

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and screening, drug delivery systems, food processing and storage, air pollution and remediation, constructions, health monitoring using nanotubes and NPs, space science material production, chemical industry, information technology, textile industry, electronic consumer production, vector and pest detection and control, automobile industry. Nano-technology produces new materials with the size of atomic scale/ super molecular scale/ nanoscale. This molecular scale is usually below 100nm in simple term it is one billionth of a meter. Nanotechnology is a field recently developed and it plays a vital role in science and technology field but yet it's not in the matured level (1, 2).

Nanotechnology uses in Medicine : National Institute of Health in USA, defined nanomedicine as "highly specific medical intervention at the molecular scale for diagnosis, prevention and treatment of disease". This nanotechnology application in medicine also immature field and few methods already in action but some techniques only imagined while most of the techniques are under the research conditions (7). Nanotechnology is used in field of medicine for drug delivery, treatment, diagnostic & monitoring techniques, bio sensors, antimicrobial techniques, cell repair and control the biological system are some of applications (8). Fiber optic technology uses to monitoring diseases. Optical biosensors used to measurement physical parameters such as pH, blood flow rate, blood oxygen levels, radiation dosage. Endoscopy in next generation will extend its capability from imaging to diagnostics and therapy using nanofiber technology. Fiber optic sensors, endoscopes nano-scale bioprobes with the rapid advance of nanotechnology (9).

Drug Delivery : Drug delivery system (DDS) is defined by national institute of health in USA as, "Formulation of a device that enables the introduction of therapeutic substances in to the body and improves efficiency and safety by the control the rate, time and place of release of drug in the body." The process of drug delivery can be mainly divided in to,

1) The administration of the drug or therapeutic product can be divided as non-invasive and invasive administration. Non-invasive administration such as oral, topical (skin), nasal, and inhalation routes. Invasion administration is injection or nanoneedle array.
2) The release of the active part of the drug by the product.
3) Transport active ingredients across the biological membrane to the target site to perform action.

DDS interface, between the patient and the drug and it may be formulation of the drug or device used to the deliver the drugs to the particular site (10, 11). The usual drug delivery systems are not up to the satisfactory level. There were many drawbacks include poor bioavailability, generate side effects, low drug loading capacity, poor ability to control the size range, plasma fluctuation of the drug levels, low therapeutic effectiveness, low in- vivo stability, low solubility, no control over the time, location and lack of target delivery to the site of action as well as some drugs are only active in a narrow range. If concentration is above the threshold level it becomes toxic, if it is low lack of therapeutic effect. These drawbacks put pressure on scientists to investigate more about new DDS and it control and determine the rate and location of drug release (12). Scientists developed NPs of the size of macromolecules such as DNA and proteins. The some developed nano-structures were smaller than diameter of a double stranded DNA (2nm). The smallest cellular form in the world is a bacteria named *mycoplasma.* Which has the size of 200nm but in comparison the largest NP is only 100nm in size. New DDS has the ability to deliver drugs to specific target cells in various areas of the body without degradation in the gastrointestinal track. It includes delivery and targeting of pharmaceutical, therapeutical and diagnostic agents by the help of NPs to the cells such as cancer cells. The ultimate goal of NP drug delivery is to improve the proper treatment diagnostics and prevention of disease (3, 7).

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The NP used in DDS contains encapsulated, absorbed, dispersed or conjugated drugs and this were able to, provide lower toxic side effects, provide multi functionality targeting, delivery and reporting ability, have high saturation solubility, drug particles resistance to settling, provide improved therapeutically index, high efficiency of drug delivery, rapid dissolution, reduces plasma fluctuation level, reduces the drug dosage, The drug directly releases to site and it is in nanosize, ultimately cut down the cost of drugs (13).

Nanotechnology increases oral bioavailability of drugs as a result of their special uptake mechanisms such as absorptive endocytosis. The NPs are also able to remain in the blood for long period and release the drugs in controllable manner to the target tissue. The self-controlling system of drug releasing helps to reduce the plasma fluctuation and minimized the side effects. The drug is incorporated in to the NP which is in nanoscale and it is easily diffused through biological membranes and cells take up these particles for the efficiency in drug delivery to site of action (14). Nanotechnology improves performance effectiveness, safety, patient adherence as well as reduces the cost compare to traditional DDS. The nanotechnology successfully used in drug delivery in the treatment of cancer, asthma, and hypertension as well diabetics. There are hundreds of various ongoing researches in this field to improve efficiency of DDS (15). Nanotechnology capable of production biodegradable, biocompatible, targeting and stimulate responsive carriers such as liposomes, nanofabricated materials (fullerenes, carbon nanotubes, silicon, silica) metals (gold, silver, iron, platinum, quantum dots) and polymers (micelles, dendrimers). The nanoparticles can obtain different shapes such as spherical, rods, wires, discs, hemispherical and ellipsoidal (16). NPs are known as a successful drug delivery materials because of it contains several properties such as, high drug carrying capacity, higher stability for the drugs inside blood steam and can travel without sedimentation and blockage, both hydrophilic and hydrophobic drugs can incorporate with the carrier, the drug conjugated NP can administration in to the body using various methods such as non-invasive and invasive administration, the drug releasing from the NP matrix can be control, NPs can easily penetrate in to the tissues such as cancer cells, NPs can take up by cell naturally via endocytosis. These properties of NP attached drug delivery method leads to improve duration of drug circulation, bio-availability of the drug and control drug releasing at the particular site. Ultimately NP with drug incorporation enhancing the ability to use highly toxic, poorly soluble, unstable drugs and maximizing patient comfort (17).

History of nanotechnology uses as drug delivery : *First Generation*- This era basic mechanism of controlled drug release was established and most drug delivery formulations were oral and transdermal administration. The effectiveness and stability was low in these drug systems (4). More than 150 years ago, Michael Faraday prepared gold particles in nanometer scale. These colloidal gold particles were conjugated with antibodies for target specific staining known as immune-gold staining. This application of gold particle considered as a precursor of recent application of gold particles in nanotechnology. In 1960s, Liposomes and polymer micelles were first prepared, however it was never referred as nano-particles until 2000. In 1970s, NPs and dendrimers were first prepared without the knowledge of nanotechnological application. In 1980s, the period reported to be the successful development of micelles as drug delivery system. And in 1990s, Block co-polymers of polyethylene glycol (PEG), PEG-Polylysine have been invented by Kataoka (14). Prior to nanotechnology revolution, in past liposomes, polymeric micelles, nano-particles, dendrimers, and nano-crystals used for drug delivery, but in the era nanotechnology, the terms were unknown.

***Second Generation*:** The modern nano-technology uses as drug delivery began when United States launched the national

nanotechnology initiatives, the world first program in the field of nanotechnology. There were various new methods introduced for drug delivery among them nanotechnology method became more efficiency and cut down drawbacks in ordinary DDS (4).

Current immature nanotechnology use microchips, carbon nanomaterials, micro needles based transdermal therapeutic systems, layer by layer assembled system and various microparicles produced by inject technology as well as previous nanocarriers were developed using co-polymers such as polyethylene glycol(PEG) and ligands. (14).

Nanoparticles : There are mainly 2 types of nanoparticles as, Organic nanoparticles {Polymers in DDS (polymeric miscalls, polymeric NPs, polymeric drug conjugates), dendrimers, nano crystals and lipid based NPs like liposomes, solid lipids} and Inorganic nanoparticles {Metal NPs (gold, silver, iron, platinum, quantum dots), Silica NPs (mesoporous, Xerogels)} (5, 16).

Liposome : Lipids are amphiphilic molecules; both hydrophobic and hydrophilic parts are included in same molecule. When it is contact with water, lipid bilayer forms as a result of unfavorable interactions by naturally or can make synthetically by mechanical agitation. A lipid bilayer will close in on itself; forming a spherical vesicle separating the external environment from an internal compartment such vesicles are named as liposomes. Liposomes are able to carry both hydrophobic and hydrophilic drugs incorporated in to their vesicle tails and heads/ aqueous core respectively. The liposomes are biocompatible, biodegradable, increase solubility of drugs; improve pharmacokinetic properties such as rapid metabolism, reduction of side effects, increase of an in-vitro and *in-vivo* anticancer activity and therapeutic index of chemotherapeutic agents (18).

The shape, surface charge, size and functional groups in liposomes can easily change according to the drug and target site. Liposomes have high efficiency and low toxicity, so it can use to deliver DNA, si.RNA, proteins, antisense oligonucleotides both hydrophobic and hydrophilic chemotherapeutic agents. Liposomes can be prepared by methods such as mechanical agitation, solvent evaporation, solvent injection, surface solubilization method. Drugs are incorporated in to the liposomes by encapsulation method and it provides various size ranges, various physiochemical properties and prevents subsequent leakage from liposomes. pH, liposome composition, osmotic gradient, surrounding environment, magnetic field and radio frequency regulates the drug release to the target site (7,18,19).

Adsorption, fusion, lipid transfer and endocytosis are the various methods which can use to interaction of liposomes with cells. Liposomes are encapsulated with various drugs such as anticancer drugs, neurotransmitters, antibiotics and anti-inflammatory drugs as shown in table 1. (7)For instance doxorubicin is highly toxic compound, when treated to the cancer patients it have the ability to affecting tumor cells as well as heart and kidney, finally using liposomes it delivered directly to the tumor cells instead of accumulate in the heart and kidney.

The drawbacks include with liposomes as low encapsulation efficiency, quickly releasing of drugs, poor storage ability, lack of tunable triggers for drug release and it release drug in to the extracellular fluids. To overcome these problems scientists modified surface of liposomes using PEG and it is act as protection layer on the surface of liposomes and it can slowdown liposome recognition by macrophages. The liposomes attached to specific proteins, antigens, antibodies, ligands or other biological substances which helps to target cell recognition for target specific drug delivery (19).

Nanoparticles based on solid lipids: There are 3 main types such as solid lipid NPs (SLN), nano structured lipid carriers (NLC) and lipid drug conjugates (LDC). SLN are composed of pure triglycerides, complex glyceraldehydes mixture

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Table 1. Several drugs and treatment which uses liposomes.

	Drug	Treatment
1)	Ampotericn B	Fungal infections.
2)	Doxorubicin	Ovarian cancer, Kaposi's sarcoma, breast cancer.
3)	Cytarabine	Lymphomatous meningitis.
4)	Daunorubicin	Kaposi's sarcoma
5)	Comptothecin	Anticancer drug.
6)	Vancomycine	Antibiotics drug.

or waxes stabilized by various surfactants. They are highly stable drug release in controllable manner, drug protection and good tolerability. The drawback is lower drug loading capacity and as a result NLC and LDC are modified. NLC produced by mixing both solid and liquid lipids and increase drug loading capacity and prevent drug deposition. LDC developed as lipophobic drug molecule and prepared by salt formation by covalent linking (18).

Nano-Crystals: The drug which needs to be injected in to the cell is produced in nano size and it can function as its own carrier. The drug particles readily water soluble as a result of it nanosize. The drug particle is reduced to the nanosize range and stabilized surface by polymeric macromolecules and nonionic surfactants. The size decreases means increasing surface area of drug. Ultimately solubility dissociation is increased and plasma concentration rises. The nanocrystals can reduce accumulation of carrier particles and directly drug can incorporate with target site. Nanocrystals become stable in aqueous dispersion without any stabilizers. These can efficiently take up by tumor cells (7, 20).

Polymeric nanoparticles-These are synthetic polymers and size ranging from10-100nm such as polyglycolic copolymers, poly [-caprolactone, poly-acrylamide, poly-acrylate or natural polymers such as albumin, geltin, alginate, collagen, DNA, chitosan, alginate.

Table 2. Several drugs and treatment which uses Nanocrystals.

Drug	Treatment
Rapamycin	Immunosuppressive
Megestrol	Anti-anorexia
Fenofibrate	Hypercholesterolemia
Aprepitant	Anti-emetic

These polymeric NPs may be biodegradable or non-biodegradable (21). These are produced using various methods such as solvent diffusion, emulsification diffusion, solvent evaporation, spontaneous emulsification and use of supercritical carbon dioxide and polymerization. Recently produced smart polymers and it stimuli to environment signals such as temperature, electrical mechanical strength, enzyme and biomolecules (22*).*

The various drug incorporating mechanisms use such as 1) Covalent bonding between drug and polymer carrier. 2) Hydrophobic interactions in-between drug and carrier 3) water filled deport for hydrophilic drug incorporation. The drug are released in to the target site by diffusion, desorption and NP erosion. The polymeric NP can deliver drugs to the site of action with minimum toxic levels and it is hydrolysis inside body to produce bio-degradable metabolite monomers like lactic acid and glycolic acid (7, 18).

Table 3. Several drugs and treatment which uses polymeric NPs.

Drug	Treatment
Carboplatin	Ovarian, head, neck, lung cancer
Doxorubicin	Wide spectrum of tumors.
Lamivudine	Anti HIV drug
Clotrimazole	Antifungal drug

Polymer Drug conjugates: Protein and peptide drugs are conjugate with polymers such as polyethylene glycol(PEG), PEG-camptothecin and it can prevent protein drug degradation in stomach and also soluble in water hence increase the half life of drugs in plasma. White blood cells unable to recognize them as foreign particles because of it conjugated polymers. Recently developed brush polymer drug conjugates by ring opening metathesis co-polymerization and it solubalized in water easily than polymer drug conjugates (7).

Table 4. Several drugs and treatment which uses polymer drug conjugates.

Drug	Treatment
L-asparaginase	Acute lymphoblastic leukemia
Adenosine deaminase	Adenosine deaminase enzyme deficiency
PEGylated IFN-á-2a	Hepatitis C

Polymeric micelles: It forms amphiphilic surfactants spontaneously associated in aqueous medium to form core shell structure. Inner core of micelle hydrophobic and surrounded by shell of hydrophilic polymers such as PEG. Hydrophobic core provide space for poor water soluble hydrophobic drugs while hydrophilic surface shell stabilizes core, prolongs circulation time and accumulate in tumor. Drug incorporated either by physical encapsulation or covalent attachment. For instance paclitaxel is drug which incorporated with polymeric micelles and use to delivery chemotherapy for cancer (7).

Dendrimers**:** Dendrimers are synthetic polymers with well defined size and structural branched chain. The chemical composition regulates the biocompatibility and pharmacokinetics. The size ranging from 1-10nm while composed of core, dendrons and surface active groups. Core provides space to drug to encapsulate and it regulates the drug releasing rate. The branched monomers called dendrons are attached to the core and surface active groups determined the physiochemical properties. Dendrimers can be synthesized from core and after the layer called as divergent process. In convergent process synthesis starts at the outer surface and it stops at core region. In-vitro and In-vivo Cytotoxicity of the dendrimers is controlled by core materials and nature of dendrimers surface. Drugs can incorporate either to internal surface/core or dendrimers surface by covalent bonds and it decide by the drug and target site. High toxicity and poor soluble drugs encapsulated to core while amount of drug can control by surface attachment. Folic acid, antibiotics, cyclic targeting peptides, PEG can attach to get high activity and specificity. For example methotrexate, doxorubicin can deliver using dendrimers to tumors and ibuprofen, piroxicam as anti-inflammatory drugs (13,18).

Carbon nanomaterials: The nano-tubes (CNT'S), C-60-fullerenes and nano-horns (CNH) uses as carbon nanomaterials. Single walled CNT'S or multi walled CNT'S are produced using layer of graphite and it has higher electrical and thermal conductivity. The surface of CNT'S are covered by amphiphilic di block copolymers, PEG layer or hyaluronic acid matrix and it helps to increase biocompatibility of carrier. The drugs are attached to carrier by encapsulation drugs to CNT'S, chemical adsorption of drugs to surface and active agents into functionalized CNT'S. The CNT'S release drugs in controllable manner by electrically or chemically and it helps to protect drugs. CNH and C-60 fullerenes also have similar properties as CNT'S. They have several immobilization techniques to attach drugs as shown in table 5 (7). The drawbacks of carbon NPs is toxicity and it depends on geometrical structure and surface molecules like carbonyl, hydroxyl.

Table 5. Several drugs and immobilization method which uses in Carbon nanomaterials.

Drug	Mode of immobilization
Gemcitabine	Encapsulation
Doxorubicin	Adsorption
Amphotericin B	Conjugation
Pt (4) prodrug-FA	Covalent amide linkages
Dexamethasone	Encasulation

Silica Materials: Xerogels and mesoporous silica NPs have higher biocompatibility, convenient functionalization and high porous matrix. Xerogels has highly porous and surface area and drug loaded by sol-gel technique. The drug releasing rate can control by changing synthesis conditions such as temperature, pressure, ratio of reagents. Phenytoin, cisplatin, nifedipine, doxorubicin, matronidazole, heparin are drugs incorporated with xerogels. Mesoporous silica nanomaterials have high surface area for drug absorption, homogenous structure. Anticancer drugs, antibiotics, heart disease drugs are delivered by mesoporous and drug releasing controlled by diffusion method (18).

Metal nano-particles (MNP): Gold, silver, iron, platinum, ceramic, quantum dots and super magnetic uses as NPs because of it shape depending optical, magnetic, electrical properties and size. Physical, chemical and green approaches uses to production of MNP's. Physical methods are evaporation condensation and laser ablation. It provides less solvent contamination and uniform distribution. Chemical method uses reducing agents such as ascorbate, sodium borohydride, tollens reagent. Biological approach uses bacteria fungi and plant species for production MNM. Magnetic NPs can control with help of external magnetic field so it able to same time reported and treated diseases (23).

Others

Nanoparticle formulation

1) *Nano-precipitation:* It is easy and quick method performed by adding an organic solution which containing polymer and lipophilic drug in to the aqueous solution in drop-wise manner under constant stirring. Co-polymer particles are highly flexible while it has both hydrophilic and hydrophobic surfaces. Inside water hydrophobic parts

Table 6. Several other NP

Other Nano particles	Description
Ethosomes	Carriers that enable drug to reach the deep skin layer/ systemic circulation
Aquasomes	Composed of nanocrystals and covered with polyhydroxyloligomeric film
Phamacosomes	Pure vesicles formed by amphiphilic drugs
Collidosomes	Bilayer structure made up of non-ionic surfactant vesicles
Nonemulsions	Submicron emulsions O/W or W/O (24)
Elastomer	Like proteins (ELP) are derived from elastin and having short hydrophobic domain with 5 amino acids. Series of ELP produce by changing temperature.
Electrospunnanofibers	Used to treatment diseases and membrane applied as topical or implanted DDS. These can orally administrate (25).
Hydrogel NP	Produced by cross-linked hydrophilic polymers. It has permeability, mechanical stability, swelling properties and network structure so control by physical conditions. These can use to transport high amount of drug to target site by external magnetic field (13).

goes inside and hydrophilic parts come outside and forms globular structure while hydrophilic drug attach to polymer outer surface and hydrophobic drugs penetrated in to core hydrophobic area. Finally this particle surface can design with ligands such as antibodies to targeting. The Size of NP can control by rate of polymer addition and by stirring speed.

2) *Emulsification based methods:* The organic phase contain drug and polymer are agitated/ sonicated in aqueous phase to form emulsified droplets.

Emulsification-solvent evaporation: Polymer dissolved in volatile solvent like chloroform and emulsified in aqueous phase. Formation NP achieve by evaporation of solvent under reduced pressure. Adjusting solvent evaporation conditions such as temperature and pressure would improve quality but slower the process

Emulsification solvent diffusion: Polymer dissolved in pre-saturated partially water dissolving solvents like benzyl alcohol. It produces oil-water emulsion droplets. The dispersed droplets diluted by large amount of water containing stabilizers. Diffusion of organic solvents out from droplets lead to condensation and formation of nanoparticles.

Emulsification Salting out: Organic solvent use is totally dissolve in water. (Ex; acetone) Polymer containing organic solvent is emulsified in aqueous phase with high salt concentration. The saturated aqueous solution prevents acetone from mixing with water. Diffusion of emulsion droplets in large amount of water result in an abrupt drop of salt concentration of the continuous phase leading to extraction of organic solvents and precipitation of NP's.

3*) Layer by layer synthesis:* It makes electrostatic interaction between oppositely charged polyelectrolytes such as polylysin, chitosan, gelatin-B complex with sodium alginate, dextran sulfate, hyaluronic acid, heparin or conbriatin sulfate. Solid form of bioactive agents is often use as core to grow the vesicular structure. A polymer layer is first absorbed on to the colloidal template by incubation in polymer solution, washed and transferred to opposite charged polymer solution. Repeat the cycle to multiple coating that can control the release kinetics. This method is used to manipulate bioactive agents such as vitamins, peptides, insulin and nucleic acid (14).

4) *Genetic engineering method (GEM):* It can control structural, functional properties of recombinant protein based drug carriers such as elastomer like proteins and silk like proteins. GEM can control molecular weight, hydrophobicity, drug conjugate site and secondary structure as well as provide higher transfection efficiency (14).

5) *Electrosprayed Technique:* The NP produce setup consist syringer pump with polymer solution connected to high voltage power supply. Metal foil collector placed opposite functions as ground electrode. Flow rate and applied voltage depend on type of solution used in process. Solid particles can produce by solvent evaporation. Electrosprayed particles can used to deliver directly drugs without polymer to the target site (26).

Mechanism of drug delivery using nanoparticles : The drug bullets are attached to NP and it contains ability to cure the diseases. The nanotechnology based DDS is only provide proper delivery of drug to target sites without any changes occurring in parental therapeutic particle. The drugs required special pH conditions, poorly water soluble or required high concentration of drugs in order to become therapeutically effective (11). Drug Polymer attachments are the encapsulation, non covalentcomplexation and conjugation to polymeric carriers via liable linker are the main methods use to attach drug to polymer. Size of polymer-drug conjugate plays major role and it should be control by adjusting the molecular weight of polymer. Drug-polymer attachment changes the drug solubility, hydrophobicity and

permeability (14). The NP's has drug loading capacity and it depends on matrix density. The drug loading capacity can increase by minimize solubility, increase ionic interactions between drug and matrix and by maximizing the absorption of drug load. Drug and polymer covalently attached via linkers and they are pH or enzyme sensitive (17,21). The drug attached NP can recognized by the immune cells and it can destroy. To overcome this problem the particle surface is decorated with biodegradable, hydrophilic copolymers to allow particles to circulate long period. The degradability could be control rate of the drug releasing rate. Poly-glycolic acid (PGA), poly-lactic acid (PLA) and their co-polymers are widely used for decorating the surface. PEG-copolymers are greater interest due to their ability to condense nucleic acid in to nano-sized polyplex with protective and biocompatible PEG shell. Moreover PEG can resist serum protein adsorption, prolonging the systemic circulation of particles, reduce toxicity (11). Ligands also attached to the NP surface to get higher specificity drug delivery to the target site. Antibodies, protein, peptides, carbohydrates, lipoproteins, charged molecules. Nucleic acid ligands like DNA, si.RNA, m.RNA, are known as apatamers and have high affinity and specificity for target (27).

Oral, intravenous, arterially, dermal, transdermal and inhalation are methods use to enter NP to the body. The Drug-NP conjugate injected to circulation system and it can take up by the calls/tissues. Drug is delivered through blood by dissolving, dispersing and finally reached to the target site. Traditional DDS circulate drug in to all the cells in body while nanotechnology based DDS provide drug to target site by their ligand attraction process. The drug-NP conjugate should able to deliver drug to target site without degradation in gastrointestinal track, without reducing drug activity and volume. Secondly it should attack to target cells without harm to other cells and reduces side effects (14,28). The drug delivery to the cells can be of 2 types. 1) Passive targeting-The drugs are diffused to the extra cellular matrix and diffused to the cell. It enhancing permeability and cellular retention effect of NP. Tumor vesicles are highly disorganized and presence of pores. So enlarge the gap junction in-between endothelial cells. These pores of tumor site allow NP to enter easily to tumor cells than normal cells. Passive targeting is not applicable to all tumors and normal cells, because some tumor cells are lack of pores. Diffuse of drugs out of NP decreases with deceasing concentration of reservoir (16,29). 2) Active Targeting - Affinity ligands, antibodies, aptamers bind to the specific receptor in the cell surface. Nanocarriers bind to the target cell through ligand-receptor interaction by the expression of receptors or epitopes on cell surface. These receptors are highly expressed on tumor cells than other cells (15,30).

The NP surface decorated by ligands and these ligands can attach with the specific receptors in the surface of targeted cell by bio-recognition. The NP's are entered to the target cells by receptor mediated endocytosis. In this endocytotic vesicle generated when segment of plasma membrane invaginate, enclosing with NPs. Thousands of NP's easily can enter to cell by this method. Inside the cell NPs are developed in to endosomes. Then endosomes merge with each other to form large endosomes or lysosomes. Finally therapeutic drugs can release in response to enzymes or acidic pH with controllable manner by degradation of polymeric NP shell (7).

Controllable drug releasing in particular sitescan be control by different ways, 1) Polymers are biodegradable and it degraded in controllable manner to release drug to site 2) Pores within the polymer can be altered in the preparation method. So drug diffusion occurs more readily or slowly. 3) The distance of fusion and surface area of the NP can alter by changing size. The size of NP also plays major role, smaller size means larger surface area. Drug releasing and drug dissolve is faster and this can control engineering by changing size of NP. The drugs

are released by matrix by diffusion, swelling, erosion or degradation. The drug releasing control by osmotic pressure, mechanical pumping and through electro kinetic transport. Constant drug releasing can achieve by tuning the properties of nano-fluidic devices (16).

Nanotechnology uses in Treatments

Cancer treatment: The usual drug delivery to the tumor cells develop side effects in normal tissues such as nephrotoxicity, neurotoxicity, cardiotoxicity and multiple drug resistance(MDR) reduces drug concentration at target location, poor accumulation. MDR is mostly due to the increase efflux pumps in cell membrane such as P-glycoprotein. Pacilitaxel loaded NP can pass drugs without disturbing by MDR (31). To overcome these problems NP based drug delivery system is used. The tumor sites forms new blood vessels to supply nutrients and oxygen rapidly. These newly formed vesicles are defective and have leaky vasculature allow NP to diffuse. The energy requirement increase and glycolysis occur. Ultimately acidic environment generated and the advantage of pH uses to drug releasing (11,32).

Nano X-ray nano-particle therapy

1) In standard radiotherapy X-ray able to hydrolysis water molecules to produce free radicals. It can ultimately damage DNA and other molecular structures in both tumor cells and healthy cells. Nano X-ray NP has self-protecting layer to minimize unwanted interactions and suspended in water. It is injected to cancer patients and it gets attached only with tumor cells by specific recognition. Nano x-ray NP attracts X-ray more readily than water. Finally it can damage both double stranded and single stranded DNA in Tumor cells to kill only tumors without harming healthy cells.

2) The NPs are attached with highly toxic cancer drugs like Doxorubin and NP surface decorated with PEG and target ligands to drug delivery to target site without harming to healthy cells (33).

3) Photothermal therapy-Au NP has optical properties and that allow absorption of light near ultraviolet. Due to the increase temperature of cell above 42°C the viability of cells are lost. Following the irradiation of the body or under magnetic field, the NP gets heated up and that leads the irradiation of tumor cells. Angiogenesis inhibition-metal particles can inhibit phosphorylation of protein involve in the process angiogenesis by binding to the cysteine residues in heparin binding growth factor (34).

4) Cetureimab, fluorouracils are drugs attached with liposomes, hydrogels, crystals to treat oral cancers and overcome low solubility, permeability and poor bio-availability (35).

5) Most applications are still under research conditions, animal testing or only an envision. Researchers try to a) Improve blood circulation period of NP by coating their surface with red cell membrane instead of PEG. b) Reduce side effects by using gold NP's for platinum cancer therapy. c) Design different N.P's with different shapes, ligands and drug particles to treat tumors. d) Using photosensitive agents that accumulate in tumor and cause blood vesicles more porous to penetrate NPs more easily. e) Attach RNA to treat skin cancers (36). f).Spherical NP Coated with si.RNA to treated lung cancers (37). g) Monoclonal antibodies and vaccines are directed against tumor (38,39).

***Heart Diseases*:** This is still under research. NP is a protein produced by translation and used to attach damaged regions of arteries as well as to break blood clots. NPs are tried to direct under magnetic field to deliver proteins to right place in arteries.

In Diabetics: Developed NP containing insulin attached to matrix. The enzymes are attached to NP, when blood glucose level increases enzymes stimulate insulin releasing and ultimately it can regulate blood glucose level for several days.

***Ophthalmic diseases*:** a) polymeric NP, nanogels, liposomes, micelles, dendrimers, chitosan and protein NP's are investigated to treat several ophthalmic applications for back of the

eye diseases like diabetic retinopathy, retinoblastoma, retinitis pigmentosa. The drug and gene deliver to the target tissue for treatment of posterior segment disorders like choroid and retina, improving diagnosis and retinal prosthesis. b). to treated glaucoma nano-diamonds with drug (timolol maleate) embedded in contact lenses (40,41).

***In Tuberculosis (TB*)**:** Treatment of TB required continuous and frequent drug supply to the cells. The NP attached with drugs such as rafampin (RMP), Inosiazid (INH)/Pyrazinamide (PZA) and covered with PEG to provide drugs sustainable manner to TB cells. Researchers try to improve bioavailability, reduce dosing frequency and drug administration methods in TB treatment (17).

***Bone diseases*:** The calcium-phosphate based NP used in drug delivery to bone diseases without any toxicity to bone tissues. Arthritis, osteoarthritis, osteosarcoma and metabolic bone cancer treat using drugs such as biosphosphonates. Silica and magnetic NP success in bone regeneration (42).

Central nerve system diseases: NP can cross blood brain barrier (BBB) so it can use to deliver drugs to brain tumors, alzheimer's disease, inborn metabolic errors like lysosomal storage disease, infectious diseases and aging etc. Most therapeutic particles are unable to pass through BBB, blood cerebrospinal fluid barrier, or other specialized central nerve system barriers. Only a small class of drugs or molecules with high lipid solubility and low molecular mass can pass through BBB. NP has high affinity and able to specifically transport drug through BBB. Some transport molecules like growth factors, insulin and transferring can increase efficiency and kinetics of drug across range of tissues (43,44).

Other ongoing researches: 1) NASA developed bio-capsules to protect astronauts from effect of radiation. 2) Try to deliver antigens to the body to enhances immune system. 3). Improve dental implant by adding nanotubes to surface of implant matrix. 4)Try to attach RNA to NP surface to improve time of circulation (45).

Future opportunities : In future nanotechnology based DDS can improve to treat in the antitumor therapy, gene therapy, radiotherapy, delivery of proteins, antibiotics, vaccines, vesicles through BBB. Before human application mechanism and fate of NP-drugs should be study using animal models scientists will be able to develop drug loading, targeting, transporting, releasing, interaction with the barriers, low toxicity and safe conditions. The understanding of drugs, when delivered to sensitive organelles like nucleus as well as able to improve NPs to treat bone diseases and bone regeneration. Multi-functional NPs might be developed that are capable of detecting malignant cell, deliver different drugs at same time, visualize the location by imaging agents, killing cancer cells with minimum side effects and monitor and treat at the same time. (13,29,42,46). There is an ability to improve this particle to cure diseases like HIV, cancer and same nanoparticles can develop as robots to operations like heart diseases. The nanoparticles can combine with computer programming system to automatically regulate homeostasis in human such as blood glucose level, ca-level. We can also improve these NPs as powerful protectors in body towards foreign particles in future.

Discussion and Conclusion

Nanotechnologies as drug delivery systems are designed to improve the pharmacological and therapeutic properties of conventional drugs. The highly toxic and low selectivity drug are transported to the target site without accumulate in any place by using nanoparticles. The nanotechnology improves bioavailability of drugs, efficiency and selectivity as well as reduces the side-effects and toxicity. Reduction of plasma fluctuation and higher solubility also play vital role in drug delivery. Various nanoparticles are used to deliver drug such as polymeric miscalls, polymeric NPs, polymeric drug conjugates, dendrimers, nano crystals and lipid based nano-particles like liposomes, solid lipids. Inorganic NPs like metal NPs (gold, silver, iron, platinum, quantum dots) and Silica NPs (mesoporous, xerogels).

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The drugs are binds to the nanoparticle by help of different conjugations like encapsulation, non-covalent complexation and conjugation to polymeric carrier via liable linkers. After the polymer surface is covered by co-polymers like PEG to get protection from immune cells. Ligands are antibodies, proteins, charged molecules, carbohydrates, aptamers are attached to get high specificity towards target side. The drug conjugate NP enters to the cell by passive or active targeting, respectively by diffusion or by receptor mediated endocytosis. Finally nanoparticles can release drugs by controllable manner in response to enzyme or pH changes. NP based drug delivery still develop to cure diseases like cancers, diabetics, heart diseases and central nerve diseases are some of them. The nanoparticle based drug delivery can be further developed to cure most challengeable diseases like AIDS in future. Nanotechnology can be developed in future to treat all type of diseases in human at the same time by producing multifunctional nano-particles.

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Vol. 10 (1) 92-107 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Plants as Potential Resources of Anticancer Drugs

Sheik Aliya[1], Yalavarthy Prameela Devi [2*] and Adepelly Uma[1]
[1]Centre for Biotechnology, Jawaharlal Nehru Technological University, Hyderabad, INDIA
[2]Professor, Department of Zoology, Kakatiya University, Warangal, INDIA.
*For Correspondence - prameeladeviy@gmail.com

Abstract

Cancer is a complex disease condition affecting millions of people all over the world. Chemotherapy and synthetic drugs lack specificity and targeted efficacy leading to drastic side effects. For thousands of years humans have been using plants to treat various diseases. Plant derived anticancer drugs are promising agents approved worldwide (approximately two-third of anticancer drugs). Many plants with active anticancer agents are identified which has high affinity to a biological target and their potency to inhibit the cancer metastasis is studied extensively. This review mainly enlists medicinal plants of whose active component with anticancer potential have been identified and applied in research and preclinical trials. The medicinal plants whose active components are yet to be identified but crude extracts exhibits cytotoxic activity against almost most of the human cancer cell lines. At last on the molecular mechanism of bioactive compounds of twelve medicinal plants which are widely used to cure different types of cancer. Knowledge of these indigenous anticancer plants forms the platform for new, safe and effective drug development.

Key words: Anticancer agents, cancer, medicinal plants, herbs

Introduction

The use of medicinal plants to cure almost any human disease is not just a custom of the distant past. Even with the development of synthetic chemical drugs for pharmacological treatment, 90% of the world's population still relies mainly on raw herbs and crude plant extracts as medicines (1). In India, out of a population of 2.1 billion, more than 70% still use these non-allopathic systems of medicine. Appropriately about 60% of anti-tumor and anti-infectious drugs already on the market or under clinical trial are of natural origin (2) (3) (4). Even The National Cancer Institute (NCI), USA has screened more than 33,000 plant samples for anti-tumour activity and nearly 55000 plants for other diseases (5). Thus, both nationally and internationally there is an increasing demand for herbal medicinal plants. It is expected that the trade of the medicinal plants will rise to 5 trillion dollars by the year 2050 (6).

For thousands of years humans have been using plants to treat various diseases (Table 1). Worldwide, with over 350,000 known plants, it is estimated that there are about 21,000 medicinal plant species. These medicinal herbs, shrubs and trees are more concentrated in the Amazon rainforest of South America, Coastal Forests of East Africa, the eastern Himalayas and Western Ghats in south Asia, and the Eastern Arc Mountains, which are known as global biodiversity 'hot-spots'. Some common medicinal plants are listed in Table 2. Plants synthesize primary metabolites (proteins, fats, and carbohydrates necessary for sustenance and reproduction) and secondary metabolites (like "antibiotics" because they protect the plants against fungi, bacteria, animals, and even other plants).Thus, secondary metabolites evolved as chemical defenses that play important roles in

combating infectious diseases and herbivore predation (7). The secondary metabolites or bioactive compounds like alkaloids, glycosides, tannins, volatile oils and minerals and vitamins, possess medicinal properties. Examples of important drugs obtained from plants are vincristrine and vinblastine from *Catharanthus roseus*, camptothecin from the tree *Camptotheca acuminate*, digoxin from *Digitalis* spp., pilocarpine from *Pilocarpus* spp., quinine and quinidine (antimalarial) from *Cinchona* spp., picrotoxin from *Anamirta* spp., atropine from *Atropa belladonna*, L-Dopa from *Mucuna* spp., sennosides from *Senna* spp. and morphine and codeine from *Papaver somniferum* (8) (9).

According to the World Health Organization (WHO), in developing countries, about 80% of people are dependent upon traditional medicines primarily because of their wide affordability, easy accessibility and cultural familiarity. In fact, as up to 40% of the world's poor have no access to the government health services, traditional and folk medicine is the only medicine available to them. In western countries, which predominantly use synthetic medicines, the pace of re-adopting the use of traditional medicinal plants is by no means uniform (7). Thus the use of herbal extracts as medicines has created a new wave in today's generation.

Issues on anticancer plants toxicity : Phytomedicines are widely accepted not only in underdeveloped or developing countries but also in developed countries. Plants are taken as food or swallowed for therapy or accidentally ingested by children or animals. But lack of general awareness of the use of medicinal plants could be potentially harmful can lead to plant toxic accidents. A popular quote of Mark Twain which states "Be careful about reading health books. You may die of a misprint." Wrongly identified plant (needs botanical identification) or using a substitute of a specific plant as a drug leads to fatal condition or toxic accident. Toxicity can result from highly concentrated doses or the form of use. *Aloe species* ("babosa") and *Euphorbia tirucalli* are commonly used for treating cancer in folk medicine. But now they are legally prohibited for internal use because of presence of high content of hydroxyanthracene glycosides, hepatotoxic pyrrolizidine alkaloids (Portaria 10/SVS, 30.01.1992) causes severe diarrhea , makes "babosa" potentially toxic (10). Plants and plant derived products are our pharmaceutical cultural heritage but they are poorly explored. Training and general awareness about the incredible plants involving the native communities exposes one to the indigenous knowledge of ancient secret traditions (5).

Screening and Natural availability of bioactive compounds : Plants are apparently the direct source of secondary metabolites compared to other sources such as marine algae (11) (12). But screening of plants for its potential property itself takes a long period of time. The NCI took nearly 25 years to screen 20,000 plant species (from Latin America and Asia) for anti-tumour activity while other pharmacological properties were not screened (13). If identified and are easily harvested through agricultural practices and are produced in bulk, while the processes that prepare them for medicinal use are time consuming. Rare and indigenous plants are available in restricted thick forest maintained as hotspots by tribal people who consider the herbs to be their riches and god's gift. However, exploitation of medicinal plants and endangered herbal shrubs complicate the drug availability issues. Earlier crude drug was used, whose effect was slow due to meager concentration of the main active ingredients. Medicinal chemistry evolution developed the technique isolating pure active compounds from the raw form of the drug. The purification of quintessential bioactive compounds with various medicinal properties from a single crude drug opened up a new page in the drug exploration chapter. However, there was a long gap between the isolation of bioactive principle (the magic bullet) and structure elucidation of the magic bullet. For example, opium was used for thousands of years for many medical purposes. Morphine, the active compound of opium, a powerful pain reliever was

purified between 1820 and1850. Still, it took years of research to modify the drug to make it less addictive (14) (15). Isolation of pure compounds from raw plant material and application of the compound or its synthesized analogues for effective results in clinical trials marked the birth of Pharmacology. In 1958, Monroe Eliot wall reported the presence of anti-cancer component in Camptotheca (Happy tree) , but it took nearly 38 years of research and several rounds of clinical trials to approve the use of synthetic derivative of Camptothecin , topotecan by US Food and Drug Administration.

Paclitaxel is an efficient anticancer drug isolated from bark of the *Taxus brevifolia* (Pacific Yew). It has shown good potency against lung, ovarian, and breast cancers, as well as several others. However, natural product-derived drug development was greatly affected due to severe supply shortages. It has been estimated that to treat a patient for a year, four full-grown 70-year-old Pacific Yew trees must be chopped down in order to recover the required paclitaxel content. This collection was at a peak in 1988 and 1989, which threatened the survival of this relatively common tree. Recently, modern techniques such as plant cell culture have been subjected and standardized to provide a steady and economical supply of paclitaxel (16). However, this is also a time-consuming process.

Bioavailability of active principles : Despite the productive application into mainstream cancer chemotherapy, the phytochemicals such as vincristine and taxol (commercial anticancer drugs), still many formulations are unsuccessful at the research level itself because of many prohibitory factors. Firstly, the complex composition of plant extracts with the lack of reproducibility of activity and secondly the synergy between different, even unidentified, components of an extract (17).

The low bioavailability of most bioactive compounds is due to factors such as metabolic instability, high physical activity and poor aqueous solubility at basic and neutral pH values. These limitations decrease the activity and therapeutic efficacy of phytochemicals, prohibiting the full utilization of medicinal plants (18) (19). Polyphenolic nutraceuticals, which are abundantly available in plant-oriented diets, undergo intestinal transformations by microbiota and enterocyte enzymes to form an active compound that protects us from degenerative diseases like cancer. Absence of microflora due to high medication makes the micronutrient worthless. Extensive research studies are being carried out to understand the causes of non-bioavailability of the drugs. Firstly lack of selectivity of most anticancer drugs against the specific cancer cells (20). Secondly single drug efficiency and availability is less compared to combination of one or two drugs. Some researchers have proved that the synergistic action of drugs neutralizes each other's negative effect and interacts with the other to enhance the anticancerous activity. Example is a soybean isoflavone derivative, Genistein, when combined with cisplatin, exhibited enhanced inhibitory activity on proliferation of HCC (hepatocellular carcinoma) cells (21) (22). Similar results of inhibitory action against prostate cancer with combination of Rg3 (an active ingredient of root *Panax ginseng*) and docetaxel was reported. Combination drug treatment of prostate cancer was proved to be better than the use of single drug (23). Etoposide, etopophos and teniposide are diverse derivatives of Podophyllotoxin (obtained from *Podophyllum peltatum*) are currently used in combination with other drugs for treatment of a variety of malignancies. But the supply and demand of natural source of drug is problematic, which calls for the need for urgent alternative sources. Other investigations have focused on the design of new analogs to make phytochemicals more effective (24) (25) (26). Zhao et al. (2013) reported a multi-targeted DNA topoisomerase II inhibitor, the HY-1 (podophyllotoxin derivatives) which induced G2/M phase arrest resulted in decreased proliferation of human colon cancer cells (27).

Plants as resources of anticancer agents : Worldwide, cancer has become a growing health problem, killing annually about 3500 per million populations (28). A large number of synthetic chemopreventive agents are used to treat various cancers, but they cause drastic side effects that result in mild to severe complications (29) (30). Despite their intricacies there is active development of more than 1500 anticancer drugs with over 500 of them under clinical trials. Thus, there is a compelling need to develop less toxic and more effective natural drugs for anticancer treatment. Kintzios and coworkers (source) performed an intensive study on plants with phytochemicals likely to possess antitumor or anticancer properties. They found 102 genera, 61 families, and a total of 187 plant species that process phytochemicals with antitumor properties. Of these species, only 15 (belonging to 10 genera and 9 families) were at the clinical level of cancer chemotherapy while the rest of the recognized plant species were either active on cancer cell lines or were reported showing anti tumor activity under experimental conditions. It was also found that the most widely distributed compounds were Phenylpropanoids (18 families), followed by terpenoids (14 families), and alkaloids (13 families) (Table 3 and 4) (17).

Epidemiological studies have revealed that populations with primarily vegetarian diets have lower incidence rates of various cancer types. Recent findings implicate a variety of phytochemicals, including monophenolic and polyphenolic compounds, flavonoids, and alkaloids, from a large variety of plant foods, spices and beverages. These compounds have been shown to inhibit or attenuate the initiation, progression and spread of cancers cells *in vitro* and in animals *in vivo*.

In this review, the focus is mainly on medicinal plants (Fig. 1) whose active compounds (Fig. 2) and their analogs are extensively used in *in vitro* and *in vivo* in clinical trials and cancer therapy. Understanding the interaction of these compounds at the molecular level magnifies the niche of the cancer cells. Thus opens up the new horizons for the further level of cancer treatment.

Agrimonia pilosa (Rosaceae): Traditional Chinese medicine regards agrimony as one of the most important herbs for cancer as its extracts can inhibit every type of cancer except leukemia and the painful bone, liver and pancreatic cancers. Agrimoniin is potent antitumor tannin that has been found to suppress the growth of MH134 and Meth-A solid type tumors. Agrimoniin has also shown strong cytotoxicity on MM2 cells in vitro, suggesting that the antitumor effect may be due to enhancing the immune response of the host animals through the actions on tumor cells and some immunocytes(31).

Angelica glauca (Umbelliferae): *Chorak,* an aromatic, perennial ornamental striking herb, has a long history of cultivation for use as a medicine, flavoring agent and vegetable. In India Angelica *glauca* is critically endangered. The Millennium seed bank partnership has aimed to preserve it for the future generation. It is found in the north western Himalayas but is highly traded due to its medicinal value. Decursin a potential chemotherapeutic agent, isolated from *Angelica gigas*, has been found to induce cell cycle arrest and apoptosis in various cancer cell types (32) (33; 34), including breast (35) and prostate cancer cells (36) . *Angelica glauca*, root has been reported to have potential anticancer properties. Decursin (Fig. 2) the compound that was extracted from Korean Angelica has become an important clue for cancer researchers after they conducted a number of studies to check its efficacy (37) (38) (39). Six coumarins isolated from the roots of *Angelica glauca* (Fig. 1), in which decursin showed stronger cytotoxic effect compared to others (40). Seung Hwa Son and coworkers (41) reported the anti-angiogenic potential of decursin. It reduced VEGF-induced angiogenesis by down regulating the phosphorylation of ERK (extra-cellular signal-regulated kinase) and JNK (c-Jun N-terminal

Table 1: Legacy of the past:

S.No:	Societies	Chronological Indications
1.	Neanderthal man, some 60,000 years ago	valued herbs as medicinal agents; Evidence is based on a grave in Iran in which pollen grains of eight medicinal plants were found
2.	Ancient Sumerians, some 5000 years ago	Plants with medicinal qualities were processed correctly, used heal illness and injuries.
3.	Ancient Egyptians, 3000 BC	compiled recipes and the process of medicine on the Ebers papyrus, for physicians reference.
4.	Indian system of medicine	The Rig-Veda a sacred Hindu text lists herbal medicines, Ayurvedic system for treatment.
5.	Ancient China	Pun-tsao text - written in the 1600s - list of herbal medicines compiled
6.	Ancient Greece, 8th to 6th centuries BC	Hippocrates (Father of medicine) sought herbal remedies as cures for illness and disease. Dioscorides work *De Materia Medica* listed 600 species of plants that had some sort of medicinal quality.

kinase) in HUVEC (human umbilical vein endothelial cells). A study performed by Quein Ahn et al (42) discovered that apoptosis was induced in KBM-5 myeloid leukemia cells by decursin. They also found that COX-2-dependent survivin pathway was down regulated.

Camellia sinensis (Theaceae): Green tea an evergreen shrub native to Southeast Asia yields both green tea and black tea (same species). Green tea is produced by lightly steaming or fermenting the leaves of *Camellia sinensis* (Fig. 1). It is rich in the class of polyphenol compounds known as catechins. Green tea is an accepted cancer prevention treatment in Japan and Fiji (43) (44). The green tea catechins (-)-epigallocatechin-3-gallate (EGCG) and (-)-epigallocatechin (EGC) have displayed strong growth inhibitory effects against lung tumor cell lines H661 and H1299 (45) (46). GTP has been shown to exhibit antimutagenic activity *in vitro,* and inhibit carcinogen- and UV-induced skin carcinogenesis in vivo. The properties of green tea polyphenols such as inhibition of UV- and tumor promoter-induced ornithine decarboxylase, cyclo-oxygenase, and lipoxygenase activities, antioxidant and free radical scavenging activity; enhancement of antioxidant (glutathione peroxidase, catalase, and quinone reductase) and phase II (glutathione-S-transferase) enzyme activities; inhibition of lipid peroxidation, and anti-inflammatory activity make them effective chemopreventative agents against the initiation, promotion, and progression stages of multistage carcinogenesis (46) (47). Molecular level analysis of cancer prevention by green tea catechins was studied using different animal models to check whether the molecules inducing apoptosis are direct targets for EGCG. Many oncogenic tumor models are studied to understand the molecular mechanism for example, In lung tumorigenesis models c-Jun and ERK1/2 phosphorylation inhibition, in colon cancer models phospho-AKT and nuclear â-catenin levels, in colon and prostate cancer models IGF/IGF1R axis inhibition, and in lung and prostate cancer models VEGF-dependent angiogenesis suppression but

the mechanism of primary action is still not proved with authentic data (48).

Cannabis sativa (Cannabaceae): Marijuana, the common name for cannabis (Fig. 1), is a plant that has been used for its medicinal properties for thousands of years. Cannabinoids (Fig. 2), the active components of *Cannabis sativa* and their derivatives, act in the organism by mimicking endogenous substances, the endocannabinoids that activate specific cannabinoid receptors. Cannabinoids exert palliative effects in patients with cancer and inhibit tumor growth in laboratory animals. They do so by modulating key cell-signaling pathways, thereby inducing direct growth arrest and death of tumor cells, as well as by inhibiting tumor angiogenesis and metastasis. Study reports CB1 and CB2 cannabinoid receptors are expressed in normal epidermis and in skin tumors and that both receptors are functional in the induction of apoptosis of skin tumor cells and the regression of skin carcinomas. Data indicate cannabinoid antitumoral action may also rely on the inhibition of tumor angiogenesis. Study data suggests local cannabinoid administration may constitute an alternative therapeutic approach for the treatment of non-melanoma skin cancer (49) (50).

Croton caudatus Geisel. (Euphorbiaceae): The common and local name of *Croton caudatus* (Fig. 1) is Alimpai, Fahra Hna and Ranlung Damdawi. It is a straggling, climbing shrub. Traditional healers in India use it for cancer, diabetes, indigestion, sinusitis, malaria, and piles. Croton is one of the largest genera of flowering plants, many species of which are widely used in ethnomedicine for the treatment of several diseases including cancer (51) (52). Leaves are claimed to have anticancer property. Prof.

Table 2: Some common medicinal plants and therapeutic indications:

Sl. No.	Plant name (common name)	Family	Traditional therapeutic indications
1.	*Thymus vulgaris* (Thyme)	Lamiaceae (Labiatae): Mint family	Slow down the ageing process by maintaining the vigor of our body cells.
2.	*Salvia officinalis* (Sage)	Lamiaceae (Labiatae): Mint family	Saga tea is an antiseptic for treating mouth ulcers and sore throats. Used for centuries to cure memory disorders and depression
3.	*Anthemis nobilis* (Chamomile)	Asteraceae (Compositae) : Sunflower Family	An excellent antispasmodic and digestive tonic and as food preservative
4.	*Allium sativum* (Garlic)	Liliaceae : lily family	Contains fungicides and is used in the treatment of Candida. It is also good for those suffering from a heart disease
5.	*Alcea rosea* (Holyhock)	Malvaceae : mallow family	The flowers are used for treating respiratory and inflammatory ailments, roots for diarrhoea, severe coughs bronchitis angina, inflammation, and constipation
6.	*Angelica archangelica* (Angelica)	*Apiaceae* (Umbelliferae) *:* Parsley family	It helps those suffering from flatulence, colic, Buerger's disease and a tonic for bronchitis.
7.	*Hyssopus officinalis* (Hyssop)	Lamiaceae (Labiatae): Mint family	Used in the treatment of chest congestion and coughs, to soothe sore throats, and to act as a mild sedative.

Potsangbam Kumar, associate professor, ethno botany and plant physiology laboratory of Manipur University affirmed *Croton caudatus Geisel* leaves for the treatment of cancer in the Saikot area of Manipur. Presence of crotoflorine, crostsparimine and sparsiflorine in leaves and dotriacontamol, bomyrin and â-sitosterol in the roots and barks of the plant, are used in treatment of ailments related to calcarious (cancer), was reported by the Central Drug Research Institute, Lucknow.

Curcuma longa (Zingiberaceae): Cúrcuma is a small perennial herb with high medicinal and cultural value in India, bearing many rhizomes on its root system, which are the source of its culinary spice known as Turmeric (*Cúrcuma - rizoma secco in polvere*) (Fig. 1) and its medicinal extract called Curcumin (*Cúrcuma extracto refinado*). In India, *Curcuma longa* has been in use as a culinary ingredient since around 3000 BC. Curcumin (Fig. 2) causes apoptosis (death) of various cancer cell types including skin, colon, stomach, duodenum and ovary in the laboratory. As of October 2011, there are more than 4300 articles cited by Pubmed on the subject of *Curcumin longa* products including 1604 on cancer. Curcumin has been shown to possess anti-angiogenic properties and these effects manifest due to down regulation of pro-angiogenic genes such as VEGF and angiopoitin and a decrease in migration and invasion of endothelial cells. One of the important factors implicated in chemoresistance and induced chemosensitivity is NF-êB. Curcumin has been shown to down regulate NF-êBand inhibit IKB kinase thereby suppressing proliferation and inducing apoptosis (53) (54).

Dionaea muscipula (Droseraceae): Darwin described the Venus flytrap as “one of the most wonderful plants in the world.” The Carnivorous, Venus Fly Trap contains powerful bioactive secondary metabolites that can prevent or cure cancer. The compounds include naphthoquinones (Plumbagin), phenolic acids (Gallic Acid) and flavonoids (Myricetin) processes potent therapeutic properties (55). Plumbagin strengthens and stimulates the immune system. It also exerts strong anti-cancer activity by quenching inflammation and causing cancer cells to self destruct. Plumbagin was effective in inducing cancer cell death with the HER2 breast cancer. Capable of generating reactive oxygen species (ROS) and inducing DNA cleavage, plumbagin inhibits topoisomerase II in HL-60 cells (56). It also shows a cytotoxic effect on non-small cell lung cancer (NSCLC) cell line A549 cells and is described as being able to disrupt the microtubular network by interacting directly with tubulin (57). Gallic Acid (Fig. 2) is a natural antioxidant and inhibits cancer cell proliferation and causes cancer cells to cell destruct. It induces cell cycle arrest at G0/G1 in human leukemia K562 cells by down-regulating cyclin D and E levels. Furthermore, this phenolic acid inhibits BCR/ABL tyrosine kinase and NF-êB pathway activity (58) (59). Myricetin (Fig. 2) is a quercetin analog a flavonol, exerts anti-cancer properties to induce apoptosis in pancreatic cancer cells *via* the activation of caspase-3 and 9 (60). It induces apoptosis of human bladder carcinoma cell line T-24, inhibits human colon carcinoma cells (61).

Hypericum perforatum *(Hypericaceae)*: Photodynamic therapy (PDT) has been described as a promising new modality for the treatment of cancer. PDT involves the combination of a photosensitizing agent (photosensitizer), which is preferentially taken up and retained by tumor cells, and visible light of a wavelength matching the absorption spectrum of the drug. Hypericin (Fig. 2), a powerful naturally occurring photosensitizer, is found in Hypericum perforatum plants, commonly known as St. John’s wort. In recent years, increased interest in hypericin as a potential clinical anticancer agent has arisen since several studies established its powerful *in vivo* and *in vitro* antineoplastic activity upon irradiation. Investigations of the molecular mechanisms underlying hypericin photocytotoxicity in cancer cells have revealed that this photosensitizer can induce both apoptosis and necrosis in a

concentration and light dose-dependent fashion (62).

Ocimum sanctum (Labiatae): Tulsi (Fig. 1) contains hundreds of phytochemicals that possess antioxidant, adaptogenic and immune-enhancing properties. *Ocimum sanctum* (OS) extract on fibrosarcoma cells in culture have exhibited anticancer activity (63) (64) .The fresh leaf of OS has been shown to enhance the immunity and also possess anticarcinogenic properties in experimental animals (65). Eugenol (1-hydroxy-2-methoxy-4-allylbenzene), the active constituent present in *Ocimum sanctum* has been found to be largely responsible for the therapeutic potentials of Tulsi in treatment of various chronic diseases, including cancer (66). Manikandan P et al. studied the combinatorial chemopreventive efficacy of *Azadirachta indica* and OS against N-methyl-N'-nitro- N-nitrosoguanidine (MNNG)-induced gastric carcinogenesis in a rat, based on changes in oxidant-antioxidant status, cell proliferation, apoptosis and angiogenesis (67). Significant antiproliferative and chemopreventive activities were observed in mice with high concentration of OS seed oil. The potential chemopreventive activity of seed oil has been partly attributed to its antioxidant activity (68) (66) (69) (70). Sun-Chae Kim and his research associates investigated anti-metastatic activity of the ethanolic leaf extracts of OS. The extract exhibited cytotoxic activity against LLC (Lewis lung carcinoma) cells. The extract significantly inhibited the activities of MMP-9 (matrix metalloproteinase-9), which plays a crucial role in metastasis of the cancer cells (71).

Vitis vinifera (Grapes) (Vitaceae): Grapes (Fig. 1) are one of the world's largest fruit crops and most commonly consumed fruits in the world both as fresh fruit (table grape) and processed fruit (wine, grape juice, molasses and raisins) (72). The skin of red grapes is a particularly rich source of a natural phytoalexin called resveratrol (trans-3, 4', 5-trihydroxystilbene) (Fig. 2). At low concentrations, it was found to exert antioxidant property. According to the National Cancer Institute's *in vitro* and *in vivo* studies, at higher doses, resveratrol exhibits cancer chemopreventive activity by interfering with different cellular events associated with all three major stages of carcinogenesis (i.e. initiation, promotion and progression) (73). Epidermal growth factor receptor (EGFR) is a type I tyrosine kinase receptor belonging to a family of receptors that also includes HER2, HER3, and HER4. Aberrant EGFR activation, mediated primarily through changes in gene amplification and autocrine stimulation, appears to be a key factor in tumorigenesis, as well as an essential driving force for the aggressive growth behavior of cancer cells (74). Grape antioxidants have been shown to inhibit expression of EGFR in head and neck squamous cell carcinoma (HNSCC) cells, which also caused an inhibition of the phosphorylation of extracellular signal-regulated kinase (ERK1/2), the highly conserved Ras/ mitogen-activated protein kinase (MAPK)-dependent pathway (one of EGFR major downstream pathways) (75). The chemopreventive activity of trans-resveratrol has been demonstrated notably in breast cancer and leukemia. Mantena and coworkers reported that the metastatic potential of 4T1 breast cancer cells was inhibited by grape seed proanthocyanidins. Ulcerogenic properties is a major disadvantage of trans-resveratrol (76).

Withania somnifera (Solanaeceae): Ashwagandha (Fig. 1), or Indian winter cherry, the extracts of which have been used for more than 3,000 years in India, is one of the main ingredients in Ayurvedic tonics to enhance health. The leaves are used in the treatment of tumors. A new dimeric withanolide, ashwagandhanolide, isolated from the roots, displayed growth inhibition against lung (NCI H460), human gastric (AGS), breast (MCF-7), colon (HCT-116), and central nervous system (SF-268) cancer cell lines, with IC50 values in the range 0.43-1.48 microg/mL (77). According to Jayaprakasam and co-researchers, Withaferin A (Fig. 2), suppresses angiogenesis, alters architecture of the cytoskeletal, and inhibits proteasomal activity. Incorporation of withanolides in the diet may prevent or decrease the growth of tumors in

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Table 3: List of plants that possess anticancer agents

Botanical name (with Hindi/common name)	Family	Parts used	Main active components showing anticancer activity
Abies holophylla	Pinaceae	leaf	Abiesesquine A
Acrorus calamus (Bach)	Araceae	Rhizome	β-asarone; lectin
Agrimonia pilosa (Hairy agrimony)	Rosaceae	root	Agrimoniin
Andrographis paniculata	Acanthaceae	Whole plant	Andrographolide
Annona Glabra	Annonaceae	Leave, bark, seeds	Acetogenins; Glabrin A, B, C and D f
Apium graveolens(Celery)	Apiaceae	seeds	Apigenin; phthalide; luteoline
Asparagus racemosus(shatavari	Asparagaceae	roots	steroidal glycosides such as shatavarin I to X
Azadirachta indica (Neem)	Meliaceae	Bark,leaf,flower	Nimbolide a limonoid
Cajanus cajan (Arhar)	Fabaceae	Leaf, seed	Cajanol, an isoflavanone
Calophyllum inophyllum (Sultanachampa)	Guttiferae	Whole plant	4-phenylcoumarins
Camellia sinensis (Green tea, black tea)	Theaceae	Leaf	catechins (-)-epigallo-catechin- 3 -gallate (EGCG) and (-)-epigallocatechin (EGC)
Citrus limon (Nibu)	Rutaceae	Fruit	Flavonoid, tangeretin, nobiletin, limonoid and limonene ; nibu
Clerodendrum infortunatum	Verbanaceae	Leaf and root	Hispidullin, cleroflavone
Croton tiglium	Euphorbiaceae	Leaf , root and bark	dotriacontamol, bomyrin and β-sitosterol
Dionaea muscipula	Droseraceae	Whole plant	Plumbagin; Myricetin; Gallic acid
Eugenia caryophyllata	Myrtaceae	Whole plant, flower bud	Eugenol
Euphorbia neriifolia	Euphorbiaceae	Leaf	triterpenoidal sapogenin
Ipomoea batatas (Sakkarkand)	Convolvulaceae	Stem (tuber)	3,4,5-tri-O-caffeoylquinic acid (polyphenols)
Mallotus philippensis	Euphorbiaceae	Stem bark Fruits	3α-hydroxy-D:A-friedooleanan-2-one β-sitosterol A polyphenolic compound rottlerin
Moringa oleifera	Moringaceae	Leaf, root	Niaziminim, a thiocarbamate
Ocimum sanctum	Labiatae	leaf	Eugenol (1-hydroxy-2-methoxy-4-allylbenzene)
Physalis angulata (Wild tomato)	Solanaceae	Whole plant, leaf	physalins B and D
Piper longum (Pippali)	Piperaceae	Whole plant	piperine
Premna serratifolia	Verbenaceae	Whole plant	acteoside (verbacoside)
Satureja montana	Lamiaceae	Whole plant	caryophyllene and α-terpineol
Semecarpus anacardium	Anacardiaceae	fruits and seeds	catechol
Taxodium distichum	Taxaceae	Seed, leaf	Amentoflavone; taxo- done and taxodione
Tecomella undulata	Bignoniaceae	bark	Lapachol is a naphthoquinone
Tylophora indica	Asclepiadaceae	Whole plant	Tylophorine
Vernonia cinerea (Sahadeyi)	Asteraceae	Whole plant	vernolide-A
Vitis trifolia	Vitaceae	Whole plant	epifriedelanol
Withania somnifera(Ashwaganda)	Solanaceae	Root , leaf	Withanolides; Ashwagandhanolide

Vol. 10 (1) 92-107 January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

Table 4: Plants with anti-cancer and anti- tumor activity

Botanical Name	Family	Parts used
Allium baketi	Liliaceae	Bulb (steroid saponins)
Alstonia scholaris (devil tree)	Apocynasaceae	Bark
Amotphophallus companulatus (Suran)	Araceae	Corm
Asclepias speciosa: (showy milk weed)	Asclepiadaceae	Whole plant
Avicennia alba	Avicenniaceae	Whole plant
Basella alba	Basellaceae	Aerial parts
Bacopa monnieri	Scrophulariaceae	Whole plant
Berberis aristata	Berberidaceae	Whole plant
Bruguiera exatistata	Rhizophoraceae	Whole plant
Bruguiera parviflora	Rhizophoraceae	Whole plant
Caesalpinia bonducella (Kantkarej)	Caesalpiniaceae	Whole plant
Caesalpinia pulcherrima	Caesalpiniaceae	Aerial parts
Carica papaya	Caricaceae	Leaf and fruit
Cassia occidentalis	Ceasalpinieaceae	Whole plant
Cedrus deodara	Pinaceae	Seed
Celtis Africana	Ulmaceae	Bark, root
Cissus quadrangularis (Hadjod)	Vitaceae	Whole plant
Curtisia dentate	Cornaceae	Stem bark
Equisetum hyemale (Common horsetail)	Equisetaceae	Whole plant
Eucomis autumnalis	Hyacinthaceae	Bulb
Glycyrrhiza glabra (mulethi)	Fabaceae	Rhizome
Gynura pseudochina	Compositae	Root
Hypoxis hemerocallidea (African potato)	Hypoxidaceae	Corm
Martynia annusa	Martyniaceae	Leaf
Pandanus amaryllifolius	Pandanaceae	Whole plant, leaf
Periploca aphyla	Asclepiadaceae	Whole plant
Pittosporum tobira	Pittosporaceae	fruits
Polygala senega	Polvgalaceae	Root
Prunus spp.	Rosaceae	Bark
Pterospermum acerifolium	Sterculiaceae	Flower
Rhaphidophora pertusa	Araceae	Stem
Seasamum indicum	Padaliaceae	Seed
Sonchus oleraceus	Compositae	Whole plant
Stevia rebaudiana	Asteraceae	Leaf and stem
Sutherlandia frutescens	Fabaceae	Stem, leaf, flower, seed
Tetrastigma serrulatum	Vitaceae	Aerial parts
Trapa natans	Trapaceae	Stem, fruit
Tricosanthes kirilowi	Cucurbitaceae	Root

humans (78). Survivin is observed to be overexpressed in tumor cells compared to normal tissue, playing an important role in both cell cycle regulation and apoptosis control. Recent studies have proven that withaferin A significantly induces apoptosis by attenuating the expression of survivin protein in the human breast cancer cell lines MDA-MB-231 and MCF-7 (79).

Zingiber aromaticum (Zingiberaceae): Lempuyang, is a beautiful ginger, originally from Indonesia. Zerumbone (2, 6, 9 humulatriene-8-one) (Fig. 2), a sesquiterpenoid from *Zingiber aromaticum* (Fig. 1), is an effective anticancer agent, possibly by its apoptosis-inducing and antiproliferative properties. The medicinal qualities of Zerumbone can be summarized as follows: suppresses free radical generation; suppresses cancer cell proliferation accompanied by apoptosis; and exhibits anti-HIV activity and anti-inflammatory. An investigation performed in HeLa and Caov-3 cell lines showed that Zerumbone reduced the secretion of IL-6 (which is an autocrine stimulator of cervical cancer metastasis) in a dose- and time-dependent manner. In ovarian cancer Caov-3 cells, Zerumbone profusely inhibited the cell cycle at G2/M phase in a dose-dependent manner (80). Zerumbone has been found to suppress the chemokine receptor CXCR4 at the transcriptional level. CXCR4, which is over expressed in various types of tumors, is potentially suppressed in a time dependent manner upon Zerumbone treatment. Zerumbone also suppressed CXCR4 induced HER2 (human epidermal growth factor receptor 2) expression in MCF7 breast cancer cell lines (81).

Recently some plants with anticancer properties are studied extensively, they are *Typhonium flagelliforme , Maytenus ovatus* and *Murraya koenigii.* The list extends every year but there is an urgent need for use of a promising high end technological laboratory work for instantaneous identification of the anticancer potential component with efficient drug properties.

Conclusion and future prospects

In India, more than 70% of the population uses herbal formulation drugs to treat various ailments, as they are rich sources of beneficial compounds and can be used in food preparation. Naturally occurring phytochemicals either extracted from medicinal herbs or found in certain diets have shown promising chemopreventive effects. However, the bioavailability of most phytochemicals makes their medicinal properties less effective. Many Indian and foreign institutions and universities are working to patent these natural bioactive components. Analogs of these compounds are designed to make it more effective principle to cure cancer. Currently, collaborative research that applies modern technology for *in vitro* and *in vivo* studies in order to analyze the mechanisms of action of cancer drug at the molecular level are being investigated. However, most of them are in the infancy stage due to lack of extensive clinical studies yet to be conducted with these agents. Cancer is not the disease of the rich, the poor class is also affected the same. The herbal drugs and Indian medicinal plants are most cost effective and practical in their translational potentials. Additionally, these naturally occurring compounds are widely available to the large portion of the population that does not have access to costlier western systems of medicine. The drastic side effects of allopathic medicine have advanced the research on herbal drugs. Therefore, this review on the anticancer properties of potential medicinal plants is critical for advancing research on “magic bullet” anticancer remedies.

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Current Trends in Biotechnology and Pharmacy
Vol. 10 (1) i - iv January 2016, ISSN 0973-8916 (Print), 2230-7303 (Online)

NEWS ITEM

SCIENTIFIC NEWS

The Prime Minister Sri.Narendra Modi has inaugurated the 103rd Indian Science Congress at University of Mysore on 3rd January, 2016. The theme of Science Congress is 'Science and Technology for Indigenous Development in India' In his inaugural speech, he has appealed the scientists to harness the full potential of traditional knowledge so that India can craft "local and more sustainable solutions" for modern-day challenges. He also urged them to focus on five Es - Economy, Environment, Energy, Empathy, Equity, and keep them at the centre of "enquiry and engineering" to make lives of people across the globe better. "If we wish to restore the harmony between human and nature, we must also harness the full potential of traditional knowledge. Societies across the world have developed this enormous wealth through wisdom gathered over the ages. And, they hold the secrets to economic, efficient and environment friendly solutions to many of our problems", said the Prime Minister. He also emphasized that the science and technology not just to make clean energy an integral part of our existence, but also to combat the impact of climate change on our lives. We have to develop climate resilient agriculture. We must understand the impact of climate change on our weather, biodiversity, glaciers, and oceans; and, how to adjust to them. We must strengthen our ability to forecast natural disasters. He has mentioned that we need to address the rising challenges of rapid urbanization. This will be critical for a sustainable world. He concluded that nothing can be a higher duty for us, whether we are in public life, or we are private citizens, and whether we are in business or explore science, than to leave the planet in a better state for our future generations. Let the different disciplines of science, technology and engineering unite behind this common purpose.

BrahMos – a Supersonic Cruise Missile

Dr.Sivathanu Pillai, a noted scientist of India at ISRO Bengaluru, on Saturday called 'BrahMos'- the Indo-Russian supersonic cruise missile, a "brahmastra for Indian Armed forces" and "a war winner". Dr.Pillai popularly known as the 'Father of BrahMos', speaking at the 12th convocation of the Dhirubhai Ambani Institute of Information and Communication Technology (DAIICT) at the convocation ceremony. On being quizzed on the need for more missiles like 'BrahMos' in light of India-Pakistan's current political situation, Pillai said, "Today if you look at BrahMos, it is the only supersonic cruise missile operational in the whole world. No other country has ventured to have supersonic system in the armed forces. We have 'BrahMos' operational in the navy in the naval fleet, we have the army on the land force and very soon it will be with the airforce. So when we

have the tri-service operation of the Brahmos, it is going to be a war winner, we have got a very good weapon available to give victory to India.

Calling India self-sufficient in space technology, the noted scientist averred that going by the demand from the world over, the next move for India should be to market its space technology. "So we are going for more number of launches in a year using the PSLV (Polar Satellite Launch Vehicle) and the trend is to go for "smaller satellites". Whereas the communication satellites are going bigger and bigger, so to suit the trend the space research program will adapt itself so that we will be able to serve the nation and also we get sizable market from the world," he added. Speaking to students, Pillai spoke about the need for a rehaul in India's education system and creation of innovation in ICT education and start-ups and knowledge system powerhouse to unleash India's knowledge potential. He also said that, "Startup and entrepreneurship has become a very important components of the education.

COP21- 2015 Paris Climate Conference

The Paris Pledge for Action is an initiative of the COP21 French Presidency. COP21, also known as the 2015 Paris Climate Conference, will, for the first time in over 20 years of UN negotiations, aim to achieve a legally binding and universal agreement on climate, with the aim of keeping global warming below 2°C. France will play a leading international role in hosting this seminal conference, and COP21 will be one of the largest international conferences ever held in the country. The conference is expected to attract close to 50,000 participants including 25,000 official delegates from government, intergovernmental organisations, UN agencies, NGOs and civil society. The world leaders including US President, welcomed the adoption of a legally-binding pact seeking to limit global warming to well below 2 degrees Celsius, calling it a big step forward in securing the planet for future generations.

Einstein's mass energy equation inadequate, claims Indian researcher

It was stated that Albert Einstein's mass energy equation (E=mc2) is inadequate as it has not been completely studied and is only valid under special conditions, an Indian researcher has claimed in an international paper. Ajay Sharma, a Shimla based researcher who challenged Einstein's derivation, he is an assistant director for education with the Himachal Pradesh government, told IANS that Einstein's theory has not been studied completely. It's only valid under special conditions of the parameters involved, e.g. number of light waves, magnitude of light energy, angles at which waves are emitted and relative velocity," he said. Einstein considered just two light waves of equal energy, emitted in opposite directions and the relative velocity uniform. There are numerous possibilities for the parameters which were not considered in Einstein's 1905 derivation, stated in Mr. Sharma's paper.

Stranding of Whales at Tamilnadu shore Thoothukudi

An unusual phenomenon in the first week of January 2016, that about 45 short-finned pilot whales dead and washed ashore between Kallamozhi and Manapad in Thoothukudi district and as many as 36 whales, part of the group which survived the beaching, were rescued in a joint operation by several government agencies and pushed back to sea. Marine scientists working in the Gulf of Mannar Biosphere Reserve say that the short-finned pilot whales are deep water

Vol. 9 (4) i - ii October 2015, ISSN 0973-8916 (Print), 2230-7303 (Online)

whales, diving up to 1,000 metres, who form stable matrilineal kinship groups. This particular group could have stranded while in search of food, the favourite being squids. Not much is known about the species. While there were rumours claiming that the beaching could be due to climate change or pollution of the waters, the scientists said the group most probably followed an isolated whale and must have been stranded. “The stranding of these whales is rare. They don’t swim close to the coast,” A similar incident has occurred in January 1973, when 147 whales were stranded on the beach between Kulasekarapattinam and Manapad, almost the same location. They survived for a few days but later all died.

2015 Hottest year on record

The National Oceanic Atmospheric Administration and NASA announced that 2015 was by far the hottest year in 136 years of record keeping. The year 2015 wasn’t just the Earth’s hottest year on record it left a century of high temperature marks in the dust. The NOAA said 2015’s temperature was 58.62°F (14.79°C), passing 2014 by a record margin of 0.29°F. That’s 1.62°F above the 20-century average. NASA, which measures differently, said 2015 was 0.23°F warmer than the record set in 2014.Because of the wide margin over 2014, NASA also calculated that 2015 was a record with 94 percent certainty, about double the certainty it had last year when announcing 2014 as a record.

SCIENTIFIC FINDINGS

Discovery of Teixobactin discovery

A new antibiotic – the first in nearly 30 years, Teixobactin, expected to beat the superbug resistance, has been discovered recently, it appears to be as good, or even better, than many existing drugs with the potential to work against a broad range of fatal infections such as pneumonia and tuberculosis. Laboratory tests have shown that the new antibiotic, can kill some bacteria as quickly as established antibiotics and can cure laboratory mice suffering from bacterial infections with no toxic side-effects. Studies have also revealed the prototype drug works against harmful bacteria in a unique way that is highly unlikely to lead to drug-resistance, one of the biggest stumbling blocks in developing new antibiotics. Such a development would represent a huge boost for medicine because of growing fears that the world is running out of effective antibiotics given the rapid rise of drug-resistant strains of superbugs and the spread of these diseases around the globe

Mapping of 'epigenome' a second genetic code

Scientists for the first time have mapped out the molecular "switches" that can turn on or silence individual genes in the DNA in more than 100 types of human cells, an accomplishment that reveals the complexity of genetic information and the challenges of interpreting it. The mapping effort is being carried out under a 10-year, $240 million U.S. government research program, the Roadmap Epigenomics Program, which was first launched in 2008.

Breakthrough for stopping HIV infection

A new artificial molecule which has managed to keep four monkeys free from HIV infection despite being injected with large doses of the virus. In essence, the AIDS virus locks onto the construct, dubbed eCD4-Ig, as though it were attaching to a cell and thus is neutralized. The Scripps Research team infected four monkeys with eCD4-Ig, forcing the monkeys’ cells to mass produce the molecule. The monkeys were then “challenged” with high doses of an AIDS virus for

up to 34 weeks. None of the animals became infected. The new study follows a similar gene therapy approach with natural antibodies that also showed promise in monkey experiments, according to AIDS vaccine researchers of Oregon Health & Science University in Beaverton.

Pluripotent Stem Cells to generate hair growth

Though common, hair loss is a distressing disorder. It can dent a person's confidence and provoke feelings of depression. Now, researchers from Sanford-Burnham Medical Research Institute in La Jolla, CA, say they are one step closer to a new treatment for the condition; they have found a way to generate new hair using human pluripotent stem cells. Transplanting hPSC-derived dermal papilla cells into mice induced new hair growth, the researchers found. Everyone will lose hair, around 50-100 hairs each day, on an average. But for some people, hair loss can be much more severe, causing partial or complete baldness. In this latest study, it has been revealed that how they effectively grew new hair using human pluripotent stem cells (hPSCs), cells derived from human embryos or human fetal tissue that can become any other cell type in the body.

Sunlight to Liquid Fuel

Specially engineered bacterium that can convert hydrogen (from the artificial leaf or another source) into alcohol-based fuel, aimed at aiming to solve a problem known to any electric utility. Capturing energy from the sun has come a long way, but how can it be stored for times when there's no sunlight. Going a step further, how can that stored energy be used for purposes other than electricity. In natural photosynthesis, biomass is produced when sunlight meets with water and carbon dioxide. Another step is typically required to turn that biomass into fuel, breaking down corn to make ethanol. A genetically modified bacterium that could bypass the biomass step and go straight to producing liquid fuel. Using the artificial leaf, they split water into oxygen and hydrogen.

Special bacterium for production of isopropanol: A special bacterium developed for production of isopropanol, an alcohol fuel comparable to ethanol. The resulting system would look like an algae form and this wouldn't need the continuous light or maintenance that in general algae require.

Telomere-Regulating Enzyme

ATM kinase, an enzyme known to be involved in DNA repair, is required for telomere elongation, according to a study published this week (November 12) in Cell Reports. The results could have implications for diverse diseases, from cancer, which is typically linked to overly long telomeres, to lung and bone marrow disorders that are associated with shortened telomeres. The researchers have devised a way to expedite the process by cutting telomeres, then looking for elongation by telomerase—a strategy that has been previously used in yeast cells. Using this method, they dubbed the addition of de novo initiated telomeres (ADDIT), the group investigated the role of ATM kinase. Blocking ATM kinase in mouse cells, then cutting down the telomeres, the group found that the enzyme was required to lengthen the chromosome caps, the result verified using the old, three-month-long method. Conversely, activating ATM kinase with a PARP1 inhibitor spurred telomere elongation.

BRCA1 Linked to Alzheimer's disease

Mice whose neurons harbor experimentally reduced levels of BRCA1, a DNA repair protein whose dysfunction is associated with breast and

ovarian cancers, suffered increased breaks in their genomes, neuronal shrinkage, reduced synaptic plasticity, as well as impairments in learning and memory, neurological problems characteristic of Alzheimer's disease, according to a study published in Nature Communications. Sure enough, the researchers also found that AD patients had greatly reduced levels of BRCA1 in their brains, compared with healthy controls. "BRCA1 has so far been studied primarily in dividing (multiplying) cells and in cancer, which is characterized by abnormal increases in cell numbers", therefore surprised to find that it also plays important roles in neurons, which don't divide, and in a neurodegenerative disorder that is characterized by a loss of these brain cells.

Stem cell therapy a hope for Multiple Sclerosis:

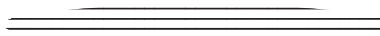
Patients with multiple sclerosis (MS) are showing remarkable improvement after receiving treatment with stem cells. Several patients have received bone marrow transplants using their own stem cells. Dramatic results in restoration of previously paralysed patients and able to walk. MS, an incurable neurological condition, causes the immune system to attack the lining of nerves in the brain and spinal cord. Most patients are diagnosed in their 20s and 30s.Now the possibility using autologous haematopoietic stem cell transplant which aims to destroy the faulty immune system using chemotherapy, and then rebuild it with stem cells harvested from the patient's own blood. These replacement cells are at such an early stage that they have not developed the flaws that trigger MS. It is presumed that the immune system is being reset or rebooted back to a time point before it caused MS.

OPPORTUNITIES

Faculty position in National Centre for Biological Sciences, Bangalore, Karnataka: NCBS is keen to consider applications from candidates with proven excellence in their field of research for faculty positions. They do not have a fixed number of posts, neither do we search for targeted research themes. If your track record demonstrates that you possess the requisite intellect, technical capabilities, commitment and potential, please do apply to us. Applications will be accepted throughout the year (www.ncbs.res.in)

Post Doctoral Positions in IIT, Delhi: IIT, Delhi invites applications from qualified Indian Nationals, persons of Indian Origin (PIOs) and Overseas Citizens of India (OCIs) for Post Doctoral Fellows (PDF) in various Departments/ Centres/Schools (in the fields mentioned along with them).The appointment will be made exclusively for research purposes and can be for a maximum period of three years (http:// www.iitd.ac.in/sites/default/files/ jobs/faculty/advt-EST-I-012014.pdf)

Postdoctoral Positions in Leishmaniasis and Tuberculosis at University of Cape Town, Cape Town, South Africa: PhD and Postdoctoral positions are available in the Division of Immunology to support the program on 'Immunology of Infectious Diseases in Africa' under the supervision of Prof. Frank Brombacher, Group Leader, ICGEB Cape Town and Dr. Reto Guler. The group performs genome-wide transcriptomics on RNA transcripts to identify host drug targets against tuberculosis and Leishmaniasis. Funding will be provided by the SARCHi/ICGEB program and the DST/NRF collaborative postgraduate training programme for two years.



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Collaboration with JNTUH, India: Alliance is having collaboration with **Jawaharlal Nehru Technological University, Hyderabad (JNTUH)**, which is a premier institution with academic and research–oriented programs, offered through the constituent and affiliated colleges. Alliance's syllabi, academic regulations and course structure are **approved by the JNTUH. JNTUH awards the degrees after fulfilling the degree requirements.**

Collaboration with University of the Pacific, USA: University of the Pacific, ranks in the top 100 among the 3000 national universities in the United States. Alliance has entered into research collaboration with Thomas J Long School of Pharmacy and Health Sciences, University of the Pacific.

Alliance students have an option to do research work at the University of the Pacific to fulfill requirements for MS degree in India. Pacific faculty teaches Alliance students via live online classes. Pacific is also interested to offer admissions to Alliance students based on their performance at Alliance.

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Registered with Registrar of News Papers for India
Regn. No. APENG/2008/28877

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Printed, Published and owned by Association of Bio-Technology and Pharmacy # 6-69-64 : 6/19, Brodipet, Guntur - 522 002, Andhra pradesh, India. **Printed at :** Don Bosco Tech. School Press, Ring Road, Guntur - 522 007. A.P., India **Published at :** Association of Bio-Technology and Pharmacy # 6-69-64 : 6/19, Brodipet, Guntur - 522 002, Andhra pradesh, India.
Editors : Prof. K.R.S. Sambasiva Rao, Prof. Karnam S. Murthy