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(Regn. No. 28 OF 2007)

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CONTENTS

Reviews

Molecular Marker Based Adulteration Detection in Traded Food and Agricultural Commodities of Plant Origin with Special Reference to Spices — 454 - 489
K. Dhanya and B. Sasikumar

Folate Receptor Targeted Delivery Systems: A Novel Micellar Drug Delivery Approach — 490 - 509
Shiladitya Bhattacharya, Xiaoling Li, Janakiram Nyshadham, and Bhaskara Jasti

Research Papers

Immunogenecity of a *Brucella abortus* S19 Glyco-conjugate Vaccine Consisting of Lipo-polysaccharide and Outer membrane Protein in Cattle Calves — 510 - 518
T. Mythili, L. Rajendra, D. Thiagarajan and V. A. Srinivasan

Biochemical Studies on the Effect of Volatile Oil of *Thymus capitatus* in Alloxan-Induced Diabetic Rats — 519 - 525
Fahim A. Benkhayal, Sara M. Al-Gazwi , S. Ramesh and Sateesh Kumar

Assessment of Rice Genotypes for Brown Plant Hopper Resistance Using Microsatellite Markers — 526 - 534
B.Vijaya Lakshmi, B.Srinivas, M.Vinay Kumar and D.Vijay

Inhibition of Tumor Growth and Angiogenesis by an Aqueous Extract of *Terminalia bellirica* — 535 - 544
Shivakumar S, Jayashree K and Bharathi P. Salimath

Microwave Synthesis of Some New Quinazolinone Formazans for their Antimicrobial and Antihelminthic Activities — 545 - 550
Rama Rao Nadendla, K.Mukkanti, G.Sudhakar Rao and A.Narendra Babu

Antibacterial Properties of Secoisolariciresinol Diglucoside Isolated from Indian Flaxseed Cultivars — 551 - 560
J. Rajesha, A. Ranga Rao, B. Madhusudhan and M. Karunakumar

Callus Induction and Antimicrobial Activity of Seed and Callus Extracts of *Clitoria ternatea* L. — 561 - 567
A.V. Mhaskar, K. Prakash, K.S. Vishwakarma and V.L. Maheshwari

High Yield Expression of Human Recombinant PTH (1-34) — 568 - 577
Srinivasa R. Gangireddy, Radha D. Madhavi, Kosana R. Ravikanth, Praveen K. Reddy, Venkat R. Konda, KRS Sambasiva Rao, Sripad Gunwar

Pharmacokinetics and *In Vitro* and *In Vivo* Correlation of NN-dimethylaminocurcumin (NNDMAC) Loaded Polycaprolactone Microspheres in Rats — 578 - 588
Jithan Aukunuru and Vidyavathi Sankavarapu

Evaluation of *in-vitro* Cultured Cells of *Withania somnifera* for Antioxidant Activity — 589- 595
Ashish Baldi, Wazid Hussain and Yogendra Tailor

Gibberellic Acid and Cycloheximide Influenced the Growth and Biochemical Constituents of a Medicinally Important Plant - *Trachyspermum ammi* (l.) Sprague — 596- 603
Haribabu Narra, Praveen Mamidala and P M Mehta

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Molecular Marker Based Adulteration Detection in Traded Food and Agricultural Commodities of Plant Origin with Special Reference to Spices

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Abstract

Plant foods and agricultural commodities including spices are increasingly subjected to adulteration by design or default, jeopardizing the age old reputation of some of the famous traded commodities and incurring heavy loss to the exchequer. The adulterants range from synthetic chemicals and earthy materials to products of plant origin. Though conventional analytical tools have good resolution power to detect the synthetic adulterants of food and agricultural commodities, these methods are hardly powerful enough to identify the biological adulterants. DNA based methods have application in biological adulterant detection and authentication of a wide range of food and agricultural commodities. This review lists some of the adulterants in powdered black pepper, chilli and turmeric and their detection with special reference to selected molecular markers (RAPD and SCAR) .

Key words: agricultural commodity, adulterant detection, food, RAPD-SCAR, spices

Introduction

Adulterant detection and authenticity testing of food and agricultural commodities of plant origin including cereals, legumes, beverages, olive oil, fruit products, spices and traded medicinal plant materials are important for value assessment, to check unfair competition and of all to assure consumer protection against fraudulent practices commonly observed in unscrupulous trade. Additionally, deceitful adulteration of these products is objectionable for health reasons, since consumption of products containing, undeclared constituents may cause intoxication or problems such as allergy in sensitized individuals (1,2).

Numerous methods, many based on morphological/anatomical characterization and organoleptic markers (odor, color, texture) or chemical testing, have been developed to authenticate troded commodity and to check for adulterants (3).

In general, the three basic detection strategies used for demonstrating adulteration in food or agricultural commodity include:

- demonstrating the presence of a foreign substance or a marker in the commodity
- demonstrating that a component is deviated from its normal level and
- demonstrating that a profile is unlikely to occur

Among these, the first strategy of detection of adulterants by the demonstration of the

presence of foreign substances or a marker is considered as the best and simplest (4,5).

The analytical methodologies/techniques used for adulterant detection or authentication of food and agricultural commodities include physical methods, chemical/biochemical methods, immunoassays and the most recent DNA based molecular tools.

Physical methods used in adulterant detection are macroscopic and microscopic visual structural evaluation and analysis of other physical parameters viz., texture, solubility, bulk density, etc. (6-11). Chemical/biochemical techniques such as high performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography (GC), gas chromatography mass spectroscopy (GC MS), nuclear magnetic resonance (NMR) spectroscopy, liquid chromatography mass spectroscopy (LC MS), liquid chromatography nuclear magnetic resonance (LC NMR), electronic nose, capillary electrophoresis polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis etc. and immunological method like enzyme linked immunosorbent assay (ELISA) have also been proved to be useful in component identification and adulterant detection in traded commodities of plant origin (12-23). However, although they are of considerable value in certain instances of adulterant detection, these methods are not convenient for routine sample analyses. Structural evaluation, which is useful for both authentication and checking for adulterants, requires expertise in analyzing the macroscopic and microscopic features of plant parts, especially those that are ground to very fine powders, mixed with other plants, or degraded due to poor storage or processing. Likewise, chemical profiling is very useful for detecting adulterants such as synthetic drugs or phytochemicals from unwanted plant material (24). Phytochemical profiles may vary on how the plant parts were processed or the environmental conditions under which the plants were grown. Furthermore, for many plant products, the marker compounds may overlap with those in related but unwanted species, or in some cases, the chemical standards may be too rare or expensive, or no marker compound has been identified for a particular botanical (3). DNA-based methods have the potential to complement these approaches (25). The use of DNA based molecular tools could be more ideal for adulterant detection in traded commodities of plant origin, especially, when the adulterants are biological substances.

Adulterant detection using DNA based methods

i. Isolation of genomic DNA

PCR based analytical methods are highly sensitive to the purity of DNA templates (26). DNA isolation from plant materials is not always simple or routine (27). Unlike the non plant DNA isolation protocols, the methods need to be adjusted to each plant species and even for each tissue due to the pleothora of primary and secondary metabolites in plants (28). Although methods are available that yield high quality DNA via binding to silica columns or beads in the presence of chaotropic salts (29-33), commercial kits that employ these methods are costly and limit their applicability. Consequently, researchers continue to modify existing inexpensive phenol-chloroform based methods, tailoring them to deal with problems such as excessive polysaccharides in specific groups of plants (34).

The modified methods in place are essentially variants of a few principal protocols viz., Dellaporta *et al.* (35), Doyle and Doyle (36), Saghai Maroof *et al.* (37) as well as Webb and Knapp (38). The modified protocols for the isolation of DNA from recalcitrant plant tissues

include those developed for seeds of sesame, soyabean, rice etc.(39); commercial samples of tea (40); cylinder of sugar cane (41); fresh and dry leaves of medicinal plants (42-44); poppy seeds (45); peanut (46); potato tubers (47); dried corn cobs (48); chick pea seeds, soybean (49); mature fresh rhizomes of ginger and turmeric (50); jams and yoghurts (51); olive oil (52,53); commercial samples of turmeric powder (54); traded cardamom seeds (55); fennel, oregano, hemp seeds, hop and dried cones (56); commercial rice, cereal products (57); fresh and dry roots of medicinal plants (58-59); dried black pepper berries (60); green and roasted coffee beans (56,61) and commercial chilli powder (62).

ii) DNA based techniques

In terms of the mechanisms involved, DNA methods are classified into three types, namely polymerase chain reaction (PCR)-based, sequencing based and hybridization-based (63).

PCR presents a high potential in adulterant detection and authentication of commodities due to its simplicity, sensitivity, specificity as well as rapid processing time and low cost (51,64,65). The PCR-based methods used for adulterant detection and authentication include the amplification using species specific primers, DNA fingerprinting methods like random amplified polymorphic DNA (RAPD) (66), arbitrarily primed PCR (AP-PCR) (67), DNA amplification fingerprinting (DAF) (68), inter-simple sequence repeat (ISSR) (69), PCR-restriction fragment length polymorphism (PCR-RFLP) (70), amplified fragment length polymorphism (AFLP) (71) and directed amplification of minisatellite-region DNA (DAMD) (72), sequence characterized amplified regions (SCAR) (73), amplification refractory mutation system (ARMS) (74), and simple sequence repeat (SSR) analysis (75). Among these, RAPD is widely used for detecting adulterants in commercial plant materials due to its low operating cost and the ability to discriminate different botanical species. Though RAPD is a fast assay in which no sophisticated technology and no previous sequence information are needed (76), it is highly susceptible to the variations in amplifying conditions (77). However, if RAPD markers are converted to specific SCAR markers, they facilitate easy, sensitive, specific aiding in the large scale screening of commercial samples for adulterants.

The development of quantitative detection strategies such as quantitative competitive PCR (QC-PCR) (78) and real-time PCR (79) have led to the quantification and confirmation of adulterants studied thereby, increasing the number of PCR applications to adulterant analysis in food tremendously.

In sequencing based methods, the variations in the species specific region of the genome (amplified rRNA genes, mitochondrial genes or chloroplast genes) due to transversions, transitions, insertions or deletions present are commonly identified (80). However, prior sequence knowledge is required for designing primers for amplification of the region of interest (79). With DNA hybridization method, detection from a variety of possible species is feasible at a time (81). However, a relatively large amount of DNA is required and the process is time-consuming (79), needs very stringent experimental conditions (81,82), and labor-intensive compared to PCR-based methods.

DNA based techniques have been applied in authentication and detection of adulteration/ cross species contamination in plant derived foods such as legumes (83-84); cereals (85-91); beverages (92,93); fruit preparations and jams

(94,95); additives such as spices (96,98); thickeners agents such as locust bean gum (99); detections of allergens (100-106) and authentication of olive oil (107-108). The applications also include adulterant detection and authentication of medicinal plant materials and products used in traditional medicine (25,63,109-115). Table 1.

Adulterants and adulterant detection in spices

International organizations like International Standards Organization (ISO) defines spice and condiments as 'vegetable products or mixtures thereof, free from extraneous matter, used for flavouring, seasoning and imparting aroma in food' (192). Traded forms of spices/spice powders are highly subjected to admixing or substitution with cheaper and inferior substances (193). The more common spice adulterants in some of the traded spices are presented in Table 2.

a. Traded black pepper

Black pepper is the most widely used spice and is often referred to as 'King of Spices'. Apart from the use as spices and flavoring agent, black pepper has antimicrobial, antioxidant, antiinflammatory and antitoxic activity (194,195). It is an essential ingredient in the Indian systems of medicine viz., Ayurveda, Sidha and Unani (196,197).

The annual trade in black pepper is valued at around 494.1 million US dollars (198). Black pepper is traded as whole dried berries and value added forms like white pepper, ground pepper/ black pepper powder, dehydrated green pepper, freeze dried green pepper, pepper oil and oleoresin (199). Pepper powder is the most common form of black pepper available to the consumer and the high process friendly nature of the commodity increases their demand in the world market. The average annual export of black pepper powder

Table. 1. Adulterant/contaminant detection and authenticity assessment of plant derived food and agricultural commodities using DNA based techniques.

Application	Technique	Target gene	Reference
Detection of cashew husk (*Anacardium occidentale L.*) adulteration in tea [*Camellia sinensis* (L.) samples	Species-specific PCR	ITS of 5S rRNA	(92)
Differentiation of 'Arabica' and 'Robusta' coffee beans	PCR-RFLP	chloroplastic genome	(93)
Detection of rhubarb yogurt in raspberry yogurt	PCR, sequencing	chloroplast *rbcL*	(51)
Detection of mei (*Prunus mume*) and plum (*Prunus salicina*) adulteration in preserved fruit products	Specific PCR	Ribosomal ITS1	(95)
Authenticity testing of raw rice materials in rice-based food product	SSR	Microsattelite DNA	(57)

Detection of basmati rice adulteration with non-basmati rice	Real time PCR	BAD2	(91)
	SSR	Microsatellite DNA	(89)
	Multiplex SSR	Microsatellite DNA	(90)
Detection of cereals and leguminous species adulteration in chestnut flour	Species specific PCR	puroindoline-a (wheat andbarley); secaloindoline-a (rye); lipid transfer protein(durum wheat, rice, maize and chickpea); thionin gene(oat); late embryogenesis abundant protein (kidney bean); lectin (soybean); nodulin (fava bean)	(116)
Simultaneous detection of wheat and barley DNA in food	Real-time PCR	PKABA1	(117)
Identification and quantification of four plant species (barley, rice, sunflower, and wheat) in food.	Real-time PCR	gamma-hordein(barley); gos9 (rice) helianthinin (sunflower); acetyl-CoA carboxylase (wheat)	(118)
Identification of durum wheat cultivars and monovarietal semolinas	SSR	Microsatellite DNA	(119)
Detection of wheat contamination in oats	Species-specific PCR	18S rDNA	(120)
Detection of wheat (*Triticum aestivum vulgare* Vill.) adulteration of spelt (*T. aestivum spelta* L.)	Species specific *PCR;*(QC-) *PCR; PCR*-RFLP	γ-gliadin gene GAG56D	(86)
Detection of soft wheat (*Triticum aestivum*) adulteration in durum wheat (*Triticum turgidum* L. var. *durum*) and durum wheat-based foodstuffs	Duplex PCR	puroindoline b; ribosomal ITS	(121)
	Species specific PCR	Pina-D1	(122)
	Real time PCR	Microsatellite DNA	(123)
	SSR/species-specific PCR/real-time PCR	microsatellite DNA	(124)

Detection of soft wheat adulteration in durm wheat and durum wheat based food stuffs.	species-specific PCR	D-genome	(85)
	Species-specific PCR/real-time PCR	gliadin, glutenin	(87)
Detection of cereal (Wheat, barley, rye, oats) contamination in gluten free foods	QC-PCR	chloroplast *trnL* intron (wheat, barley or rye)	(125)
	Real-time PCR	ω-gliadin (wheat);ω -secalin (rye), hordey (barley); avenin (oat)	(126)
	Species-specific PCR/real-time PCR	ω –secalin (rye); chloroplast *trnL* (rye)	(127)
Detection of potentially allergenic hazelnut(*Corylus* spp.) residues in foodstuffs	Species-specific PCR	Cor a 1.0401	(128)
	PCR-ELISA	Cor a 1.0401	(100)
	PCR/PNA-HPLC	Cor a 1.0301	(103)
	Real-Time PCR	*hsp1*	(106)
	Real-Time PCR	Cor a1.04	(105)
	Species specific PCR	Cor a 1.0301,	(129)
Detection soybean allergen in processed foods	Species specific PCR	Gly m Bd 30K	(104)
Detection of potentially allergenic peanut (*Arachis hypogaea*) in foods.	Real-Time PCR	Ara h 2	102,130)
	Real-Ttime PCR	Ara h 3	(131)
	Duplex PCR/PNA array	Ara h 2	(129)
Detection allergenic Buckwheat (*Fagopyrum* spp.) in food	Species specific PCR	ITS and 5.8S rRNA	(132)
Detection of walnut residues in food	Real-Time PCR	Jug r2	(133)
	Species specific PCR	matK	(134)
Detection of macadamia nuts *(Macadamia integrifolia or M. tetraphylla)* in food.	Real-Time PCR	vicilin precursor	(135)
Detection of allergenic celery (*Apium graveolens*) in food	Real-Time PCR	mannitol dehydrogenase	(136,137)

Detection of alergenic celery in food.	Species-specific PCR	mannitol dehydrogenase	(138)
Detection of allergenic mustard (*Sinapis alba*, *Brassica juncea*, *Brassica nigra*) in food.	Real-Time PCR	2S albumin	(137)
Detection of allergenic sesame (*Sesamum indicum*) in food	Real-Time PCR	*sinA*	(137)
Detection of adulterant in traded turmeric powder	RAPD	-	(97)
Detection of adulterant in traded chilli powder	RAPD	-	(62)
Detection of adulterant in traded black pepper powder	SCAR	-	(98)
Detection of adulterant in traded oregano	RAPD	-	(76)
Identification of cinnamon (*Cinnamomum cinnamomum*) from its adulterants (*Cinnamomum cassia*, *C. zeylanicum*, *C. burmannii* and *C. sieboldii*).	Sequencing; SSCP	*trnL-trnF*	(139)
Detection of origin and authenticity verification of virgin olive oil.	SSR/Real-time PCR	microsatellite DNA	(140)
	PCR, SNP/ LDR–universal array	-	(141)
	Real-Time PCR	plasma intrinsic protein	(108)
	SSR	microsatellite DNA	(142-144)
	SCAR	-	(145)
	AFLP/RAPD	-	(52)
	RAPD	–	(146)
	SSR/Sequencing	microsatellite DNA	(147)
Adulterant detection and authentication of medicinal *Panax* species	RAPD	-	(148)
	RAPD	-	(149)
	SCAR	-	(150)
	PCR-RFLP	ITS1-5.8S-ITS2	(151)
Identification of *Panax* species in the herbal medicine preparations	RFLP; PCR	-	(152)
Authentication *Panax* species	MARMS	*trn*K; 18S rRNA	(153)

Authentication of *Panax* species.	AFLP; DAMD	-	(154)
	SCAR	-	(155)
Discrimination of the Chinese drug "Ku-di-dan" (herba elephantopi) and "Pu gong ying" (herba taraxaci) from its adulterants	RAPD	-	(156,157)
Identification of the sources of medicinal Coptidis rhizome (*Coptis* species) in market	RAPD	-	(158)
Determination of the components in herbal prescription	RAPD	-	(159)
Discrimination of two very closely related medicinal plants *Anoectochilus formosanus* and *A. koshunensis*	RAPD	-	(160)
Detection of adulterants in medicnal *Echinacea* species	RAPD	-	(161)
Discrimination of medicinal *Echinacea* species viz., *E. angustifolia*., *E. pallida* and *E. purpurea*	RAPD	-	(162)
	SCAR	-	(163)
Discrimination of medicinal *Melissa officinalis* at their subspecies level.	RAPD	-	(164)
Discrimination of closely related dried *Scutellaria* plants viz., *S. galericulata*, *S. lateriflora* and *S. baicalensis*;	RAPD	-	(165)
Discrimination of medicinal *Amomum villosum* samples from their adulterants	RAPD	-	(166)
Discrimination of medicinal *Lycium* species from their closely related species	RAPD	-	(167)
Differentiation of *Lycium barbarum* from its common adulterant, *Lycium chinense* var. *potaninii*.	SCAR	-	(168)
Identification of *Atractylodes* plants in Chinese herbs and formulations	RAPD	-	(169)
Species identification in powdered plant materials of the genus *Cimicifuga* and *Trifolium*.	RAPD	-	(170)
Discrimination of *Aloe arborescens* from its adulterants.	RAPD	-	(171)
Authentication of five medicinal *Derris* species	RAPD	-	(172)

Authentication of *Mimosae tenuiflora* bark.	RAPD	-	(173)
Determination of the components in an Ayurvedic herbal prescription, "Rasayana Churna".	RAPD	-	(174)
Authentication of *Dendrobium officinale*.	ISSR	-	(175)
Authentication of *Dendrobium loddigesii*	ARMS	nrDNA ITS	(176)
Identification of *Phyllanthus emblica* in its commercial samples and multi component Ayurvedic formulation.	SCAR	-	(177)
Detection of adulteration in traded *Phyllanthus* material in crude drug (dry leaf powder).	SCAR	-	(178)
Identification of true *Sinocalycanthus chinensis* in the seedling market	SCAR	-	(179)
Discrimination of medicinal *Artemisia princeps* and *Artemisia argyi* from other *Artemisia* plants	SCAR	-	(180)
Identification of ginger (*Zingiber officinale*) from crude drugs and multicomponent formulations.	SCAR	-	(181)
Authentication of medicinal *Embelia ribes*	SCAR	-	(182)
Identification of traded medicinal plant *Pueraria tuberose* from its adulterants.	SCAR	-	(183)
Differentiation of medicinal plants *Euphorbia humifusa* and *E. maculata* from their adulterants	Real-Time PCR	rDNA ITS1	(184)
Identification of *Ephedra sinica* dietary supplements such as plant mixtures and tablets/ capsules	Sequencing	*psb*A-*trn*H	(185)
Discrimination of medicinal *Swertia mussotii* from related adulterants.	Sequencing; species specific PCR	ITS	(186)
Authentication of *Pinellia ternata* and its related adulterants	PCR, PCR-SR	mannose-binding lectin	(187)
Discrimination of the Chinese medicinal material *Gekko gecko* from its adulterants	Spececies specific PCR	mitochondrial 12S rRNA	(188)
Authentication of *Alisma orientale* and its adulterants	PCR-RFLP; ARMS	ITS; nrDNA	(189)
Discrimination of *Saussurea lappa* from its adulterants	Sequencing	ITS ;5S rRNA	(190)
Discrimination of *Dryopteris crassirhizoma* and its adulterant species	Sequencing	cpDNA *rbcL*	(191)

from different pepper producing and re exporting countries is approximately 32.4 thousand metric tons worth 99.5 million US dollars i.e., about 12 % of the total global pepper export (198).

The high commercial value of the black pepper is accountable for its adulteration (200). Black pepper berries are often reported to be adulterated with cheaper plant material of similar colour, size, and shape (201-203). Undetected adulteration of black pepper berries can lead to adulteration of the value added products such as black pepper powder and oleoresin (204).

Dried papaya seed (*Carica papaya* L.) is one of the most common adulterants of whole black pepper. Ripened papaya seeds resemble black pepper in color, size and shape (7,201,205). The addition of the seeds to the pepper berries increases the bulk of the sample

Table 2. Common adulterants in some of the major traded spices

Commodity	Adulterants	
	Chemical / earthy material	**Biological**
Black pepper berries (*Piper nigrum*)	mineral oil	Dried papaya seed (*Carica papaya); wild Piper* Spp. (*P. attenuatum* and *P. galeatum*); fruits of *Lantana camara* and *Embelia ribes;* seeds of *Mirabilis jalapa;* berries of *Schinus molle;* exhausted black pepper; light berries, stems and chaff of black pepper.
Black pepper powder	Dye	Powdered papaya seed; wild *Piper* berries; *Lantana camara*; *Embelia ribes; Mirabilis jalapa seeds; Schinus molle* berries; exhausted black pepper and light berries; starch from cheaper source
Chilli fruits (*Capsicum annuum*)	Dyes, mineral oil	-
Chilli powder	Dye- coal tar red, sudan red, para red; vanilyl- n-nonamide ; Mineral oil; talc powder; brick powder; salt powder.	Powdered fruits of 'Choti ber' (*Ziziphus nummularia*); red beet pulp; almond shell dust; extra amounts of bleached pericarp, seeds, calyx, and peduncle of chilli; starch of cheap origin; tomato wastes.
(Turmeric power. Curcuma longa)	Dye- Metanil Yellow, Orange II lead chromate; chalk powder; yellow soap stone powder.	Wild *Curcuma* spp- *C. zedoaria* Rosc or 'yellow shotti' syn. *C. xanthorrhiza* Roxb. ('Manjakua') or *C. malabarica;* starch from cheaper source; saw dust.
Ginger (*Zingiber officinale*)	Lime, capsaicin.	Exhausted ginger (volatile oil extracted).

Ginger powder	Lime	Capsicum, grains of paradise; turmeric; exhausted ginger fortified with falvours; Japanese ginger (*Zingiber mioga*).
Cardamom fruits (*Elettaria cardamomum*)	Small pebbles	Orange seeds; un roasted coffee seeds.
Cardamom seeds	-	Seeds of *Amomum aromaticum, A. subalatum* and *A. cardamomum*
Cardamom seed powder	-	Powdered cardamom hulls
Nutmeg (*Myristica fragrans*)	Pieces of clay for repairing broken nutmeg	Wild species- Macassar (*Myristica argentea*), Bombay nutmeg (*M. malabarica*) and *M. otoba*
Mace (*Myristica fragrans*)	-	Bombay mace (*Myristica malabarica*); Macassar mace (*M. argentea*).
Clove	Magnesium salt, sand, earth	Exhausted clove (volatile oil extracted); stem and fruits of clove.
Cinnamon bark	-	Cassia (*Cinnamomum cassia*)
Cinnamon powder	Eugenol, cylon oil, yellow brown dye	Aromatized and powdered beechnut husk; hazel nut; almond shell dust.
Cassia bark (*Cinnamomum cassia*)	-	Bark of *Cinnamomum japonicum*, *C. mairei*, *C. Burmannii.*
Allspice powder (*Pimenta dioica*)	-	Powdered clove stem; berries of *Myrtus tobasco* and *Lindera benzoin*
Aniseed	Fine earth materials	Hemlock fruit; parsley ; dill fruit
Aniseed powder	-	Fennel
Star anise (*Illicium verum).*	-	*Illicium anisatum* fruit
Star anise powder	-	*Illicium anisatum* powder
Nigella seeds (*Nigella sativa*)	-	Onion seeds
Caraway (caravum carvi)	-	Cumin;*Carum bulbocastanum*

Fennel	-	Exhausted or partially exhausted fennel fruits; stem tissue and stalks of fennel; umbelliferous seeds.
Mustard seed	-	Argemone seeds (*Argemone mexicana*); rape seed; ragi
Mustard seed powder	-	Added starch; turmeric
Poppy seed (*Papavar somniferum*)	-	Rajeera seeds (*Amaranthus paniculatas*)
European dill	Terpenes	Indian dill
Ajowan	Earthy materials	Exhausted ajowan seeds; excess stem and chaff.
Mediterranean oregano	-	*Origanum majorana* ; *O. syriacum*; *O. Vulgare*; *Satureja montana*.
Asafoetida	Coal tar dyes; gypsum; red clay; chalk.	Foreign resin- Gum arabic, gum resin colophony, galbanum, moriacum, resin, rosin; Barley; wheat or rice flour; slices of potato
Saffron (*Crocus sativus*)	Synthetic dyes- tartrazine, ponceau 2R, sunset yellow, amaranth, orange GG, methyl orange, eosin and Erythrosine; oil; honey; glycerine; solutions of potassium or ammoniumnitrate; sodium sulphate; magnesium sulphate; barium sulphate; borax.	Different parts of the saffron flower itself (styles, stamen, strips of the corolla); dried petals of safflower and Scotch marigold; calendula; poppy; arnica; onion skins; turmeric; annatto; stigmata from other species of *Crocus*, pomegranate, Spanish oyster and maize; dyed corn silk; meat fibre; red sandal wood; turmeric powder; paprika powder.
Vanilla beans	-	Tonka beans (*Dipteryx odorata*); *Dipteryx oppositifolia*; vanillon (*Vanilla pompona*); little vanilla (*Selenipedium chica*); leaves of orchid *Angreacum fragrans* and *Orchis fusca*; ladie's tresses (spiranthes cernua); 'vanilla-plant'(*Trilisa odoratissima*); 'herb vanilla' (*Nigritella anguistifolia*) and common sweet clovers (*Melilotus spp.)*
Vanilla extract	Synthetic vanillin, ethyl vanillin, veratraldehyde, piperonal, vanitrope and coumarin	-

and have deleterious effects upon consumption by people. Sareen *et al.* (206) and Das (207) observed the toxicity and anti fertility activity of ripe papaya seeds. Black pepper is also reported to be substituted with berries of wild *Piper* species like *P. attenuatum* and *P. galeatum* which are cheaply available as non timber forest produce. Dried fruits of *Lantana camara*, *Embelia ribes,* seeds of *Mirabilis jalapa,* and berries of *Schinus molle* (208-211) are the other minor adulterants reported in black pepper. Low quality exhausted pepper, light berries, stems and chaff of black pepper can also form as adulterants in whole black pepper (212). Coloured starches from cheaper source were also reported as adulterant in black pepper powder (202,213).

Pruthi and Kulkarni (7) developed a technique for the detection of papaya seeds in black pepper berries emplyoing the flotation test flollowed by visual and microscopic examination of the floaters. The papaya seeds and light berries of black pepper are floated in ethyl alcohol of specific gravity 0.8 to 0.82 at 25/22 degree Celsius while the mature black pepper berries sank. Bhatnagar and Gupta (6) as well as Sredharan *et al.* (8) described the utility of staining techniques and microscopic examination for the detection of papaya seed in black pepper.

The advantage of different chromatographic behavior and UV characteristics of the phenolics isolated from papaya seeds was used by Hartman *et al.* (214) for their detection in powdered black pepper. Curl and Fenwick (215) developed a test based on the determination of benzyl glucosinolate, a compound specific to papaya seed using gas chromatography to determine papaya seed adulteration in back pepper.

Paradkar *et al.* (204) and Paramita *et al.* (216) have suggested the utility of thin layer chromatography analysis in detecting the adulteration of black pepper powder with ground papaya seed. Fluorescent bands observed at 366 nm at *Rf* 0.172 and *Rf* 0.943 in the super critical carbon dioxide and ethylene dichloride extracts, respectively were identified as papaya specific markers. Jain *et al.* (211) studied the fluorescence characteristics and HPLC finger prints of black pepper and two market samples along with the common adulterant papaya seed and other minor adulterants such as seeds of *Embelia ribes* Burn. and *Lantana camara* L. Black pepper petroleum ether extract under 365 nm exhibited lemon yellow flouresecne in contrast to papaya seed with blue fluorescence.

Smith *et al.* (217) suggested that crude fibre, d-glucose, MgO, MgO: d-glucose ratio, and MgO: crude fibre ratio as the most valuable criteria for detecting the adulteration of ground black pepper with added black pepper shells. The variation in starch concentration could be used to estimate the amount of light berries present in black pepper (218).

The only DNA based report available on adulterant detection in black pepper is the development of a specific, sensitive and reproducible sequence characterized amplified region (SCAR) marker to detect papaya seed powder adulteration in traded black pepper powder (98). This specific SCAR marker could detect papaya seed adulteration in two branded market samples of black pepper powder. (Fig.1) Dhanya (219) developed SCAR markers for the detection of wild *Piper* (*P. attenuatum* and *P. galeatum*) berries in black pepper powder.

b. Traded chilli

Chilli, the dried ripened fruits of *Capsicum annuum* (Family, Solanaceae) is extensively used in all types of curried dishes in India and even abroad (220). Apart from its use as spice, chilli preparations are used as counter irritants in

Fig. 1: Amplification of papaya seed specific SCAR marker in genuine black pepper samples, commercial samples of black pepper powder and papaya seed. Lane 1-4 are genuine black pepper samples viz., 'Panniyur-1', 'Karimunda', 'Wayanadan' and 'Malabar pepper', Lane 5-12 are commercial samples of black pepper powder, Lane 13- papaya seed, Lane 14- negative control, M- 1 Kb DNA ladder (Biogene, USA).

lumbago, neuralgia and rheumatic disorders besides in the treatment of asthma, cough and sore throat (221). Capsaicin extracted from chilli is fast becoming a number one plant based pharmaceutical in the world due to its benefits as a pain reliever and a neutraceutical owing to its natural anti oxidant properties (222).

The prominent producers of chilli globally are India, China, Pakistan, Korea, Mexico and Bangladesh (223). In the year 2004, total world imports of capsicum reached 371,000 tons valued at US dollar 590 million, of which China and India's exports contributed US dollar 140 million and US dollar 94 million, respectively, for quantities exceeding 85,000 tons each (198).

Chillies are exported as dry whole fruits, crushed chilli, chilli powder and its value added products like fermented chilli, chilli paste, oleoresin etc. (199). In recent years, the global demand for chilli powder has steeply increased mainly due to their convenience in use (224). Chilli powder is the most important ground spice exported from India (221). It is estimated that around 20-30 percent of chilli crop in India is used for powder preparation. India exports around 22000 tons of chilli powder per year (199).

Compared to whole dried chilli, chilli powder and paste are more vulnerable to adulteration as foreign substances go in to it visually undetected (225). Artificial colours such as coal tar red, sudan red, para red etc., synthetic pungent compounds, brick powder, talc powder are the non plant based adulterants reported in chilli powder (213,226,227). The analytical techniques employed for the detection of artificial colour includes solid phase spectrophotometry (228); paper

chromatography (226); thin layer chromatography (229,230); gel permeation chromatography (GPC); liquid chromatography tandem mass spectrometry interfaced with electrospray ionization (GPC LC ESI MS/MS) (231); capillary electrophoresis (232); high performance liquid chromatography (HPLC) (233); polarographic method (234), UV (235); chemiluminescence (236) and mass spectroscopy (MS) (227.237,238). Todd *et al.* (239) narrated TLC procedures for the separation of synthetic pungent substitutes in chilli. Adulterants such as brick powder and soapstone in chilli powder can be easily separated based on their difference in density (213).

Dried and powdered fruits of 'Choti ber' a cheaply available red coloured fruit of the shrub *Ziziphus nummularia* Burm. (62), dried red beet pulp (240,241) and almond shell dust (241) etc. are the major extraneous plant based adulterants reported in chilli powder. Chilli powder may also be adulterated by adding extra amounts of bleached pericarp, seeds, calyx, and peduncle of chilli to increase the bulk without visibly affecting the appearance (242,225). Apart from these, the presence of starches of cheap origin and tomato wastes are also reported in chilli powder (242,244).

Schwien and Miller (240) reported microscopic examination, paper chromatography and spectrophotometric analysis for the detection of dried red beet pulp in capsicums. Pruthi (244) and Konecsni (245) described the utility of microscopic techniques in the determination of adulterants like tomato waste and added starch in chilli powder. Cox and Pearson (246) reported that a comparatively low level of non volatile ether extract as an indicative to the addition of exhausted capsicums in chilli powder.

The possibilities of the sensitive molecular tools are not much exploited in the detection of adulterants in chilli. Lekha *et al.* (247) used ISSR PCR and FISSR PCR makers for differentiating four disputed chilli seed samples, a case of marketing of spurious seeds of chilli in the brand name of an elite variety, referred to them from an Indian court of law, for varetal identification.

The utility of RAPD primers for the detection of plant based adulterants viz., dried and powdered fruits of 'Choti ber', dried red beet pulp and almond shell dust, in marketed chilli powders was described by Dhanya *et al.* (62). Comparative RAPD profiling of genuine chilli, market samples and the adulterants could identify markers specific to the adulterants. These markers were further converted to more reliable SCAR markers (219). The SCAR markers developed could detect adulteration of traded chilli powder with that of 'Choti ber' powder in one out of six samples studied.

c). Traded turmeric

Turmeric (*Curcuma longa* L. syn. *C. domestica*) belongs to the family Zingiberaceae and it is the rhizomes which are traded in different forms. It is generally used as a spice in its ground form, turmeric powder, prepared from the processed rhizomes (248). The major use of turmeric world wide is for domestic culinary purpose (249).

Besides the use as a spice, turmeric is now gaining importance globally as a mighty cure to combat a variety of ailments as the rhizome is credited with molecules having anti-inflammatory, hypocholestremic, choleratic, antimicrobial, antirheumatic, antifibrotic, antivenomous, antiviral, antidaibetic, antihepatotoxic, anticancerous properties and insect repellent activity (250). It is extensively used in Indian and Chinese systems of medicine (250-252).

India stands as the leading producer and exporter of turmeric with an annual production of around 716.84 thousand tons and export of around 46500 tones valued US dollar 382.5 million (253). About 61% of the exported Indian turmeric is traded as turmeric powder (199). Turmeric is a spice probably most subjected to adulteration since it is frequently sold in ground form (210). Govindarajan (254), Purseglove *et al*. (255), Pruthi (256), Singhal *et al*. (210) and Pruthi (257) have reviewed the adulteration of turmeric and turmeric powder.

The non plant based adulterants in turmeric powder include artificial colours such as Metanil Yellow, Orange II and lead chromate which are detected by colorimetric, chromatogarphic or spectrophotometric techniques (213,258,259). The presence of chalk powder and yellow soap stone in turmeric powder can be detected by simple chemical reaction (213).

Turmeric powder is frequently adulterated with rhizomes of cheaply available related species (210) especially with those containing the colouring pigment curcumin (255,260). The related *Curcuma* species which are of real significance in adulteration are, *C. zedoaria* Rosc or 'yellow shotti' syn. *C. xanthorrhiza* Roxb. ('Manjakua') and *C. malabarica* (97,250,261-264). *C. zedoaria* starch, 'shotti' is reported as toxic in nature (265).

The quality of turmeric is attributed to the presence of total curcumin content, which can be measured rapidly by simple spectrophotometric determination (266). Though the marketed turmeric powders are shown to have acceptable curcumin levels, they were found to be adulterated with powders from wild *Curcuma* species (97). The determination of variation in pigment composition of different *Curcuma* rhizome using TLC, spectrophotometric and capillary electrophoretic techniques have been adopted for distinguishing C. *domestica* from its adulterant *C. xanthorrhiza* (261,267). However, the study has been reported to have many limitations as the pigment content is often extremely low (268). The qualitative differences of the essential oils of turmeric and related species (262-264) were also tried as a criterion for differentiating these plant based adulterants.

Microscopy does detect the adulteration of cheaper vegetable substances in turmeric (269), but when the adulterants belong to the same genus the genuineness of the sample is difficult to decipher even by experts in microscopy as the starch grains and oleoresin cells are destroyed by boiling the rhizome (255).

Analysis based on 18S rRNA gene and *trn*K gene sequences in *Curcuma* species is found to be helpful in species identification (270). Komatsu and Cao (271) reported the variability in chloroplast *trn*K nucleotide sequences for the identification of five *Curcuma* species including turmeric (*C. longa*). Application of single nucleotide polymorphism (SNP) analysis based on species specific nucleotide sequence was developed by Sasaki *et al*. (272) to identify the drugs derived from turmeric (*C. longa)* and other related species such as *C. zedoaria, C. aromatica* and *C. phaeocaulis.*

Syamkumar (273) used RAPD and ISSR markers along with 18S rDNA sequences for the identification and authentication of Indian *Curcuma* species including culinary turmeric.

Minami *et al.* (274) performed molecular analysis based on polymorphisms of the nucleotide sequence of chloroplast DNA (cpDNA) for species identification of dried *Curcuma* rhizomes. The polymorphism observed in the intergenic spacer between *trnS* and *trnfM* (*trnSfM*) could

distinguish *C. longa* from the other three species, *C. zedoaria, C. aromatica and C. xanthorrhiza*.

Sasikumar *et al*. (97) used RAPD markers for adulterant detection in traded turmeric powder. RAPD profiles of genuine turmeric (*C. longa*) and the adulterant *C. zedoaria* were compared with three branded market samples of turmeric powder to identify the adulterant specific bands. The method could detect the admixing of *C. zedoaria* powder in all the three market samples of turmeric powders tested. Recently Dhanya (219) developed SCAR markers to detect presence of *Curcuma zedoaria* adulteration in commercial samples of turmeric powder. Using these markers presence of *C. zedoaria* or its synonymous entity, *C. malabarica,* could be detected in four out of six market samples analysed (Fig. 2).

Fig. 2: Amplification of *Curcuma zedoaria / C. malabarica* specific SCAR marker in pure turmeric, commercial samples of turmeric powder and *C. zedoaria* and *C. malabarica*. Lane 1-4 are turmeric cultivars /varieties viz., 'Alleppey', 'Amalapuri' 'Prathiba', 'Sudarshana', Lane 5-10 are commercial samples of turmeric powder, Lane 11-*C. zedoaria*, Lane 12- *C. malabarica*, Lane 13-Negative control, M-1 Kb DNA ladder (Biogene, USA).

Conclusion

Spices assume special significance as they are high value export oriented commodities extensively used for flavouring food and beverages, in medicines, cosmetics and perfumery. Synthetic substances as well as natural products are used as adulterants. Adulteration is also a major economic fraud involving public health. The Sanitary and Phytosanitary regulations of the WTO at the international level make the issue very critical and significant especially with the exported commodities. The Food Safety and Standards Authority (FSSA) of India at the national level and the Food Safety Commisionerates (FSC) at state level are also set up/being set up realizing the gravity of the issue.

The worldwide spice market was worth US $ 2973.9 millions and a corresponding 1547.2 thousand metric tonnes were globally exported in 2004, outlining a steady upward trend (198). However, the quality of spices is a major concern at present both at export and domestic trade, as the commodity is of high value traded in low volume. Unlike the whole commodity, powders are more amenable to adulteration as the foreign matters go in to it visually undetected. Spice adulterants come in different forms. In addition to artificial colors, powdered plant based materials of cheap origin as adulterant are currently on the rise especially in spice powders like black pepper, chilli and turmeric. Though advanced chromatographic/spectroscopic techniques are available for easier detection of the chemical adulterants, the plant based adulterants are more difficult to detect. A few microscopic/ chemoprofiling techniques so far developed for their detection have not been found discriminative enough, warranting more precise tools. Of late the cheaper availability of biomolecular assays make the employment of quick, precise and

reliable PCR based techniques affordable in a large number of food related applications. RAPD-SCAR markers are now available for the detection of plant based adulterants in traded black pepper, chilli and turmeric powders which need to be further extended as a quantitative analytical tool to regale the regulatory agencies/quality control laboratories. The ongoing development of quantitative DNA-based methods using Real Time PCR could enable in the future a quantitative analysis of species composition in mixed plant materials and products.

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Folate Receptor Targeted Delivery Systems: A Novel Micellar Drug Delivery Approach

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Abstract

Cancer is a pathological condition characterized by uncontrolled proliferation of cells that invade surrounding tissue and metastasize to new sites in the body. This disease is difficult to treat since cancer cells, unlike bacteria or virus, do not contain molecular targets completely foreign to the body. The goal of any therapy is to treat the affected tissue with minimal damage to normal tissues. With the current cancer therapies, this is often difficult to achieve as the drugs are cytotoxic in nature and often causes widespread damage to normal tissues as well. Thus, a need for targeted therapy for cancer has evolved in recent times. This review details a novel targeted micellar drug delivery approach that involves targeting of drugs and drug delivery systems to cancer cells that specifically expresses folate receptors on the cell surface. This review describes the synthetic design approach, and the ability of folate labeled amphiphilic system to form micelles which can be used as targeted drug carriers to cancer tissues.

Key words : Cancer, Chemotherpy, Radiation Phagocytosis

Introduction

The goal of a treatment regime against cancer is to eradicate all cancer cells from the body or at least bring them down to such a number that the patient might outlive the time required for a relapse of the disease. This can be accomplished in a number of ways. An obvious strategy is to surgically remove the cancer that is only possible when the tumor is localized, the tumor has not invaded the neighboring tissues and the mass of tissue to be removed can be partially replaced by the body to maintain homeostasis. Surgery is often complicated by the fact that tumors may grow at certain anatomically critical or inaccessible sites and the tumor cells may be extensively intermingled with healthy tissue. Radiation therapy is another alternative to treat tumors. Proliferating cells in the G2/M phase are highly susceptible to damage by radiation since they do not have enough time for DNA repair (1). Thus healthy tissues with a rapidly dividing population such as bone marrow, hair follicles, gastrointestinal tract and oral mucosa also get affected during radiation therapy and show various symptoms of acute toxicity. Apart from this, healthy organs that fall in the path of radiation but do not have a rapidly dividing cell population also get affected over time and may cause reduction in the dose of radiation to be given to patients over their lifetime. This is due to the fact that these organs require a longer time to recover. Yet another approach to tumor mitigation is chemotherapy. Similar to radiation therapy proliferating cells are susceptible to cytotoxic drugs and conventional chemotherapeutic agents kill cells by disrupting the cell division or by DNA damage. Their action is non-specific and may

cause serious damage to healthy cells. Thus, in both these paradigms, the therapeutic window is narrow and the dose given to a patient relies heavily on the dose limiting toxicity experienced by the patient that arises due to non specific cell kill from the treatments. This essentially forms the desired features a delivery system that is designed to target specifically cancer cells while doing minimal harm to normal tissues. Targeted delivery was originally proposed in the early 20th century by a German scientist Paul Ehrlich. This idea, called magic bullet, was developed from his desire to create compounds that selectively target the disease causing organism while sparing the normal tissues.

Mechanism of Tumor Targeting

The physical basis for tumor targeting lies in the fact that the tumor vasculature is more leaky than in normal tissues (2). Thus macromolecular drug conjugates get into the tumor by diffusion, convection and transcytosis in an exchange vessel. Among these routes of entry, diffusion is considered to be the major route of transvascular transport as the interstitial fluid pressure of the tumor is high due to high vascular permeability and low lymphatic drainage (3, 4). The drug conjugates targeted to tumors in this fashion are classified under passive tumor targeting. The submicron size range of drug delivery systems is often used to target tumor tissues passively by enhanced permeation retention effect. Since tumor tissues have leaky vasculature, the delivery system escapes from the circulation into the tissue yet cannot drain back into the circulation due to high hydrostatic pressure in the vessel. The delivery system needs to be in circulation for a considerable amount of time is needed for both active or ligand dependant targeting as well as passive targeting. At present, Doxil®, pegylated liposomal formulation of doxorubicin and Abraxane®, nanoparticles formulation of paclitaxel, are examples of passively targeted chemotherapeutic agents. On the other hand, tumor cells not only differ in physical aspects from normal tissues but they also express different levels of pro-survival proteins that promote growth (5-7). The different levels of these proteins serve as biomarkers of cancer and are targeted for therapeutic purposes and are more commonly referred to as active targeting. The common mode of uptake of any drug delivery device in active targeting is by receptor mediated endocytosis (8). It has also been observed that functional inhibition of certain biomarkers in cancer leads to tumor cell death (9-11). Thus the targeted tumor therapy currently encompasses both the fields of active tumor targeting and chemotherapeutics that specifically target one or more biomarkers to elicit tumor cell death. The scope of this article is limited to active targeting and further discussions will be limited to active targeting of chemotherapeutics.

Design Principles of Targeted Delivery Systems

A targeted delivery system consists of a homing device connected to a delivery system which carries a payload of the drug. The homing device is usually a small molecule ligand or an antibody for a receptor to which the delivery system is targeted. Since antibodies to a target protein are highly specific they make good homing devices. The nature of the delivery system depends on the physicochemical properties of the drug and the ligand, the regional constraints of the target and the time for which the delivery system needs to be available for action. In some cases, the drug may be directly attached to the homing device. Reactive functional groups on the drug are often utilized for making conjugates of drugs and the homing device. In such a system, after the drug reaches the target, it must be cleaved from the homing device to exert its action as conjugation to the homing device often results

in loss of pharmacological activity of the parent molecule. Insertion of acid labile linkers or cleavable peptide sequences is often used to tag drugs to homing devices so that they can be released later in the cell. Labeling a delivery system with the homing device constitutes another method of targeted delivery. The drug is either physically entrapped in the delivery system or chemically conjugated with it. Examples of such systems include drugs encapsulated in targeted liposomes or nanoparticles and targeted drug polymer conjugates. Homing devices are conjugated directly or via spacers to the delivery systems. The spacers often provide with reactive endgroups that are used for conjugation reactions or they may act to reduce steric hindrance offered to the homing device and target interaction by other components of the delivery system. Common spacers used in delivery systems include polyethylene glycols, whose terminal hydroxyl group is substituted by an amino or a carboxylic acid group, ethylene diammine and short alkyl dicarboxylic acids. The common elimination pathways for macromolecular delivery system are elimination by phagocytosis by macrophages and by the reticulo endothelial system. Phagocytosis can be minimized by the use of polyethylene glycol coating on the delivery system. The coating makes the system more acceptable to biological systems and thus evades phagocytosis. Some common approaches to targeted delivery involving cell surface receptors are illustrated in Figure 1.

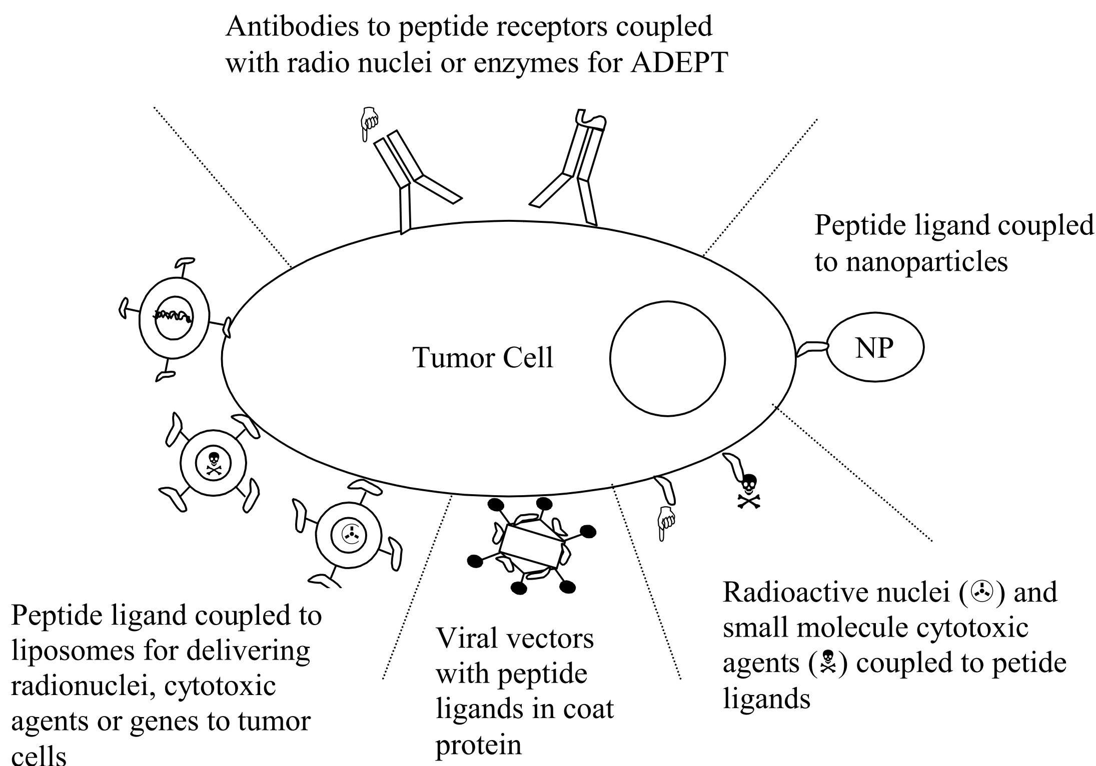


Fig. 1: A summary of strategies used for targeting chemotherapeutics to tumors

The Folate Receptor

The discovery of the folate binding protein in the human placenta provided clues for a method of site specific drug delivery (12-16). The folate binding protein also called the folate receptor is a 38.5 kD glycoprotein protein with a high affinity [$kD = 10^{-9}$ M] for folic acid (12). The receptor may be lost from the cell surface by the activity of a metaloprotease and is found to be excreted in human or bovine milk (17). The receptors are also linked to the cellular growth kinetics and are found to be less expressed in slowly growing cells or when a colony reaches confluence (18). The genes encoding this receptor are located on chromosome 11q13 at the FGF3 locus (19). The folate receptors are diffusely distributed on the cell surface but multimerize by binding to secondary antibodies and are concentrated in the caveolae (20). After binding to folic acid, the receptor is internalized and is recycled back to the cell surface after dissociation from the substrate (21) (Fig. 2). This caveolar concentration of the receptors is also controlled by cholesterol and the internalization takes place by a non-endocytic process (22). The receptor mediated pathway of folate uptake is regulated by intracellular levels of folic acid (23). Although the folate receptor is found widely distributed in the body (24), the folate receptor-alpha is over-expressed consistently in non-mucinous ovarian carcinomas and tumors of epithelial lineage in endometrium, lung, breast, renal cells and brain metastases (25). Thus the therapeutic advantage of targeting the folate receptors is due to their over-expression, often twenty times more, in these types of malignancies than in epithelial cells or fibroblasts (24). Current approaches that utilized folate receptor in targeted delivery systems are listed in Table 1.

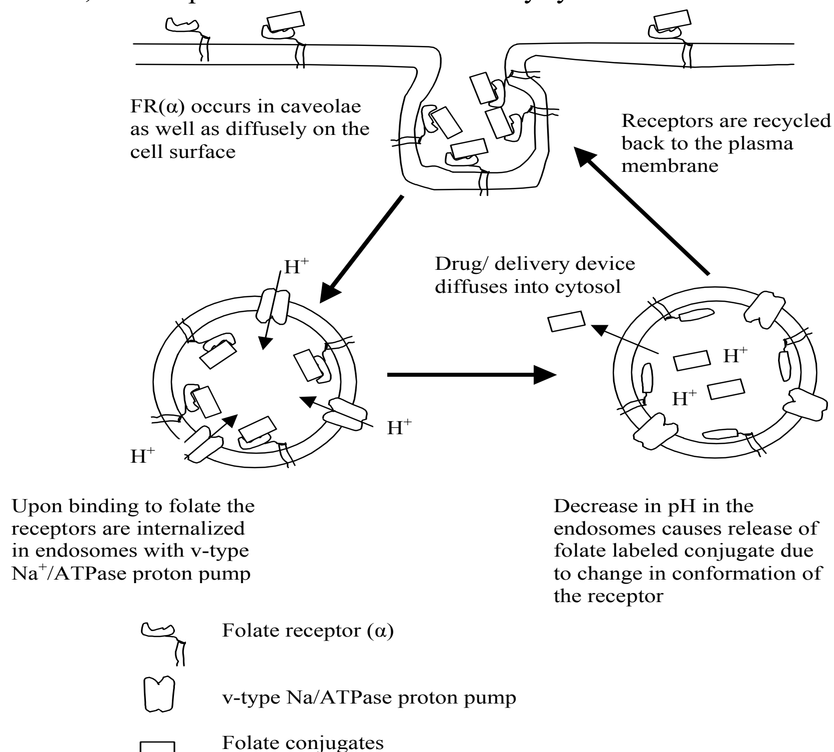


Fig. 2: Intracellular trafficking of folate receptors

Table 1. Current approaches that utilized folate receptor in targeted delivery

Polymer	Drug	Delivery system	Ref
PLGA-TPGS-Dox+TPGS-Fol	Dox	Nanoparticle	(26)
Fol-peptide-imaging agent	Pyropheophorbide	Conjugate	(27)
Poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide-co-undecenoic acid)-Fol	Taxol	Polymeric micelle	(28)
Fol-PEG-PLGA	Dox	Polymeric micelle	(29)
Fol-PEG-OligoDN-GFP	Gene	Polymeric micelle	(30)
Fol-poly histidine-PLLA		Polymeric micelle	(31)
Fol-PEG-PANAM G3.5	Indomethacin	Dendrimer	(32)
Fol-PANAM G5		Dendrimer	(33)
Fol-PAMAM	Methotrexate	Dendrimer	(34-36)
Fol-PEG-DOX	Doxorubicin	Nanoparticle	(29)
Fol-PEG-chitosan	Gene	Nanoparticle	(37)
Fol-BSA	Protein	Nanoparticle	(38)
Fol-Chitosan	DNA	Nanoparticle	(39, 40)
Fol-PEO-PPO-PEO/PEG	Taxol	Nanoparticle	(41)
Fol-Penicillin G amidase	Phenacetyl-Dox	FDEPT (ADEPT)	(42)
DPPC/DMPG/mPEG-DSPE/folate-PEG-DSPE	Taxol	Liposome	(43-48)
Desacetylvinblastine monohydrazide-Fol	Desac etylvinblastine	Conjugate	(49)
Polyether polyol-PEG-Fol	Tamoxifen	Dendrimer	(50)
Fe oxide-PEG-Fol		Nanoparticle	(42, 51, 52)
Thioctic acid-PEG-Fol on Au nanoparticles		Nanoparticle	(53)
Fol-Solid lipid nanoparticles	Hematoporphyrin, taxol	Solid lipid nanoparticle	(54)
Fol-PEG-Polycaprolactone	Paclitaxel	Nanoparticle	(55)

Fol-(PEG3350)-distearoyl-phosphatidylethanolamine	Doxorubicin, siRNA, aclacinomycin A	Liposome	(38,40,56-58)
Fol-PEG + poly(2-(dimethylamino)ethyl methacrylate)	DNA	Polyplex	(59)
(99m)Tc-picolylamine monoacetic acid folate	Tc99	Conjugate	(60)
DTPA-PEG-Fol , desferroxamine folate	Tc99	Conjugate	(61-66)
Shell crosslinked nanoparticles	^{64}Cu	Nanoparticle	(67)
Fol-^{18}fluorobenzylamide	^{18}F	Conjugate	(60)
Fol-spacer-drug	Gemcitabine	Conjugate	(68)
Fol-thymidylate synthetase inhib		Conjugate	(69)
Fol-peptide-camptothecin	Camptothecin	Conjugate	(70, 71)
Fol-RNA	siRNA	Nanoparticle	(72)
Fol-PEG-beta cyclodextrin		Conjugate	(73)
Fol-PEG PT	Carboplatin	conjugate	(74)

Micellar Drug Delivery System

The interaction of oil films on water surface has been well documented. But the interaction of hydrocarbon chains in the bulk of water was theorized principally by J. Traube in late nineteenth century. He noted that a long hydrocarbon chain attached to a polar group tends to migrate to the surface of water rather than stay in the bulk of the solution. Their presence at the surface of liquid can be measured by the decrease in surface tension which is linear at very low bulk concentration of the solute. At high concentrations of the solute the decrease in surface tension loses this linear inverse relationship and begins to saturate. It is observed that at low concentrations of an amphiphilic solute the ratio of the surface concentration of the solute to that of the concentration in bulk increases threefold for addition of one methylene group to the hydrocarbon chain. Such a relation also exists in homologues series of other amphiphilic molecules. Thus the cause of the observed effect is due to the lack of affinity of the water molecules for the hydrocarbon chains. Measurements of the free energy of attraction of water and hydrocarbons yield a value of -40erg/cm^2. The free energy of attraction of hydrocarbons for themselves is also about -40erg/cm^2 at the same temperature whereas, for water molecules the free energy of attraction is -144erg/cm^2. Thus it is the strong attraction between water molecules that supports the avoidance of water hydrocarbon interactions or the hydrophobic effect. The hydrophobic effect can be explained from a mechanistic point. Water itself is a highly structured liquid due to the presence of hydrogen bonds between water

molecules. For the dissolution of hydrocarbons in water some of these bonds must be broken in order to accommodate the hydrocarbon core. But at the same time the water molecules at the surface of the cavity formed by hydrocarbons in the bulk of the solution arrange themselves in order to regenerate the broken hydrogen bonds thereby, creating regions of higher degree of local order than present in pure water producing a decrease in entropy. An increase in the concentration of amphiphilic hydrocarbons in water will thus require the formation of hydrocarbon water interface resulting in a large decrease in entropy. It has been observed that the change in enthalpy ($H_{mic} - H_w$) for amphiphilic hydrocarbons is nearly zero for ionic and/or zwitterionic micelles and is positive for nonionic micelles hence the driving force for micelle formation, observed with an increase in the concentration of the amphiphile, solely arises from a positive entropy change. The hydrophobic effect drives micellization but the repulsion of headgroups limits its size. It is this balance of the two opposing forces that result in the formation of micelles as opposed to phase separation and are characterized by discrete aggregation number rather than a statistical size distribution (75). Some commonly used amphiphilies employed to construct micellar systems are listed in Figure 3.

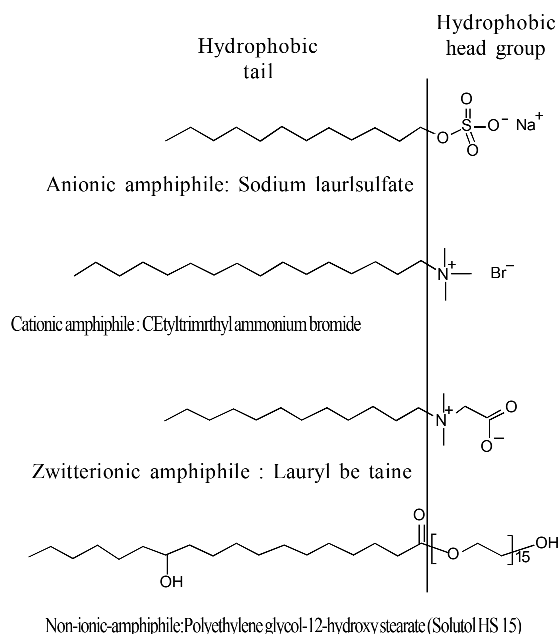


Fig. 3: Examples of different classes of amphiphiles classified according to their charge of the polar headgroup

One of the important applications of micellar systems is their solubilization capacity of poorly water soluble compounds. Solubilization of a poorly water soluble compound via micelles of an amphiphile is found to increase linearly after the critical Micellar concentration (cmc) has been reached (76). Micellar solubilization is analogous to partitioning of hydrophobic compounds between water and oil phases. It differs only in the fact that the micelles which compose the oil phase are dispersed in water resulting in clear homogeneous solution. The solutions are thermodynamically stable, but are sensitive to dilution if the concentration of the surfactant falls below the cmc (77). Thus the lower the cmc value of a surfactant, the more stable are its micelles towards dilution. This factor assumes importance in the formulation aspects of amphiphile drug blends used for parenteral administration as these undergo several folds of dilution in blood. As discussed previously, the micelles have a hydrophobic core and a hydrated hydrophilic shell, the loci of solubilization of drug molecules in the micelles thus varies with the degree of hydrophobicity of the solute (78). Compounds may be adsorbed at the micelle water interface or may be dissolved in the hydrocarbon core (Fig. 4). When adsorption takes place at the micelle water interface the solubility rises to a greater extent than when solubilization takes place at the hydrocarbon core (79). The shape factor of the micelle also influences the amount

of drug it can solubilize. Depending on the balance of the head group repulsion and the hydrophobic effect from the tails, micelles tend to adopt a range of shapes from spherical to more ellipsoidal or disk like and in some cases rods and worm like shapes have also been observed. As the shape of the micelles deviate from the sphere to more disk like or rod like shape, the volume of the core region relative to that of the shell increases. Thus, solubility of drugs which tend to be dissolved at the core increases as the micellar shape deviates from sphere. In case of ionic amphiphiles the ionic strength plays an important role in determination of size and cmc in water. It is generally observed that an increase in the ionic species results in lower cmc and larger micelles. Solubilization of weakly ionic drugs by amphiphiles is often due to interaction of oppositely charged species. This is observed at a certain pH condition, the drug and the amphiphile acquire opposite charges and the drug is adsorbed onto the oppositely charged hydrophilic shell (80).

One of the well known applications of amphiphiles in amphiphile mediated drug delivery is that of Taxol®. Due to the poor solubility of paclitaxel in water which is 0.6 mM (81), it is formulated in cremophor EL and ethanol. Cremophor is a mixture of surfactants made from pegylated lipids derived from castor oil. Prior to administration it is diluted with water for injection and administered parenterally. The presence of the amphiphile, cremophorEL, prevents the drug from precipitation when diluted. The micellar solution results in altered pharmacokinetic profile of the drug than that observed for the free form. It has been suggested that the altered profile is due to formation of micellar carriers of the drug in systemic circulation (82). The use of amphiphiles in parenteral drug formulations suffers from a major drawback of toxic side effects which is partly due to the high levels of the amphiphiles that are used in the formulation to counter the effect of dilution of the solution in blood. CremophorEL is known to give rise to hypersensitivity reactions, abnormal lipoprotein patterns, aggregation of erythrocytes and peripheral neuropathy. Amphiphiles constructed from RGD-fatty acid conjugates were found to enhance the solubility of paclitaxel by 87% and their miexed micelles with commercially available Pluronics were found to increased the solubility to 2.12 (μg/mL) (83)

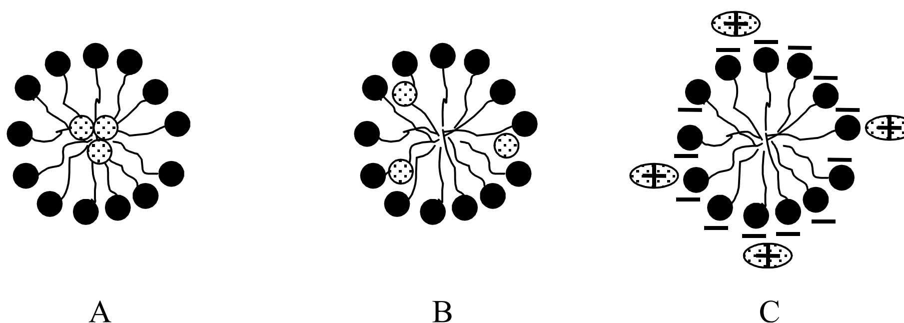
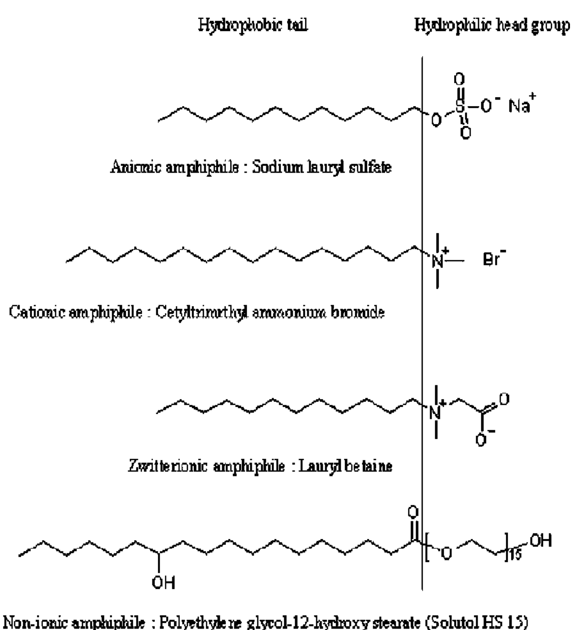


Fig. 4: Sites for drug solubilization in micelles

Folate Labeled Micellar Drug Delivery System

In recent years, targeted drug delivery has become the method of choice in cancer chemotherapeutics due to their overwhelming non-specific tissue toxicity. One of the preferred targets for active targeting of chemotherapeutics is the folic acid receptor subtype α commonly referred to as FRα. This receptor is overexpressed in ovarian and endometrial cancers and has a high affinity for folic acid. Conjugation of drug moieties and drug delivery systems to folic acid offer a route to target cancer cells overexpressing FRα. The folic acid molecule bears a glutamic acid residue coupled via its amino group to pteroic acid. The carboxylic groups of the glutamic acid residue provide a site for conjugation of folic acid residue to a number of drug delivery platforms like polymeric micelles, nanoparticles, microparticles and bioconjugates. The regiospecific conjugation of the gamma carboxylic acid of the glutamyl moiety is preferred over either alpha conjugation or a mixed conjugated product of both alpha and gamma carboxylic acid groups as alpha conjugation reduces the affinity of the folate moiety towards its receptor (85). Conjugation of folic acid when performed with the usual amide coupling reagents like DCC, EDC and CDI usually result in a mixture of alpha and gamma products which are difficult to separate. Another synthetic scheme which offers regiospecific conjugation starts with the synthesis of a specifically gamma conjugated glutamic acid moiety which is then coupled to pteroic acid. A major drawback of this procedure lies in the procurement of the expensive pteroic acid. Conversion of folic acid to pyrofolic acid and later substituting the pyroglutamic acid with desired gamma derivatized glutamic acid analogs provides a feasible method to the preparation of specifically gamma carboxylic acid derivatized folic acid analogs. The synthesis of various classes of folate labeled amphiphiles studied are summarized in Figure 5.



R = $-C_9H_{19}$ (**10[a]-FPC-9, 9[a]-FDC-9**), $-C_{10}H_{21}$ (**8[a]-FC-10**), $-C_{11}H_{23}$ (**10b-FPC11**), $-C_{12}H_{25}$ (**9b-FDC-12**), $-C_{13}H_{27}$ (**8b-FC 13, 10c-FPC-13**),$-C_{14}H_{29}$ (**9c-FDC-14**), $-C_{15}H_{31}$ (**10d-FPC-15**), $-C_{16}H_{33}$ (**8c-FC-16**), $-C_{17}H_{35}$ (**10e-FPC17**), $-C_{18}H_{37}$ (**8d-FC-18**)
$-CH2N^+(CH3)2C12H25$ (**10f-FPC-12**)

Fig. 5: Summary of the synthesized amphiphiles with major intermediates

Micellar characteristics of Folate labeled amphiphiles

Micelle formation is regarded analogous to phase separation. But unlike phase separation, the formation of micelles occurs over a narrow critical range of concentration and it is customary to assign a single concentration in this transition

zone as cmc. The cmc determination is based on a change in slope when an appropriate physical property that can distinguish between micellar and free amphiphile is plotted against total concentration. Various physical properties of amphiphilic solution such as osmotic pressure, solubilization of hydrophobic compounds, surface tension, light scattering intensity, turbidity and molecular conductivity change with increasing concentration of amphiphile as it approaches cmc (Fig. 6). To study the cmc of the synthesized amphiphiles pyrene fluorescence was used as a probe for microenvironment polarity. Pyrene is suited for this purpose as its monomer fluorescence has a long lifetime of 450 ns and it can efficiently form eximers. It is one of the few fused aromatic hydrocarbons that show significant vibrionic bands in its monomer fluorescence spectra in solution phase. In the absence of any solvent interactions, the relative intensities of these vibrionic bands in the spectrum are governed by relative potential energy levels of the excited singlet states relative to the ground state singlet and by Frank–Condon principle. The pyrene monomer fluorescence spectrum is considerably perturbed with the change of solvent from *n*-hexane (non-polar) to acetonitrile (polar). The major contribution to these perturbations is believed to be from specific solute-solvent dipole-dipole coupling. The pyrene monomer exhibits five distinct vibrionic bands of which the third shows maximum variations in intensity relative to the first band and hence the ratio of intensity of the third to the first (I3/I1)is taken as a measure of perturbation (Fig. 7). This prominent solvent dependence of the vibrionic fine structure is utilized in fluorescence probe studies of micellar systems. Pyrene is a hydrophobic probe with a logP of 6.0 and a solubility of 2-3 μM in water. In the presence of micelles and other macro-molecular aggregates pyrene is solubilized in the hydrophobic domains of these systems. Below the cmc of the amphiphiles, pyrene exhibits a I3/I1 ratio of ~0.5, similar to that observed in water. As the amphiphile concentration is raised above the cmc, pyrene is solubilized in the hydrophobic interior and the I3/I1 ratio rises. The change in the microenvironment to non-polarity is also sensed by increase in the fluorescence life time of the pyrene monomer. Since both the lifetime and the I3/I1 ratio are a function of the microenvironment around the probe, both the parameters show sharp breaks of their slope with respect to total amphiphile concentration at the cmc and indicates the onset of micellization (Fig 7).

CMC values for the folate labeled

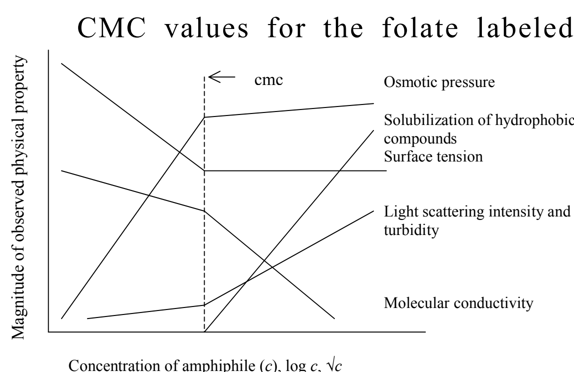


Fig. 6: Changes in the magnitude of some observed physical properties of amphiphilic solutions below and above cmc values

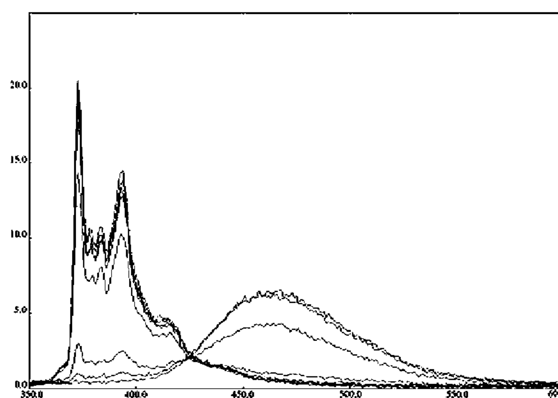


Fig. 7: The stacked fluorescence spectra show a typical change in the vibrionic pattern of pyrene fluorescence in the presence of amphiphiles. The arrows indicate spectral shifts with increase in the concentration of the amphiphile.

amphiphiles were determined by pyrene fluorescence method. In surfactant solutions, above the CMC, inclusion of more than one pyrene molecule in the micellar core gives rise to an additional band at 480nm due to the formation of an excited dimer often referred to as eximer (Fig 7). Since the α-carboxyl group of the glutamic acid was free in the final compounds, an alkaline pH of 8.4 was used to solubilize the amphiphile and the CMC of the molecules were determined at this pH. Increasing the pH further would make it unsuitable for biological studies and the amphiphile did not have sufficient solubility, neither for analytical studies nor for biological experiments, at any pH lower than this. It was observed that the cmc of the amphiphiles decreased with the increase in the hydrophobic chain length in a homologous series (Figs. 8-10 & table 2). The cmc of compounds bearing more than twelve carbon atoms in the first series FC(n) could not be measured due to very poor solubility of the compounds even at alkaline pH. The cmc(s) of all the other synthesized amphiphiles are listed in table 2.

Table 2. CMC (s) of the synthesized amphiphiles and their yield

Amphiphile	Compound number	Critical Micellar Concentration (µM)
FC10	**8a**	37
FC13	**8b**	21
FDC10	**9a**	50
FDC13	**9b**	40
FDC15	**9c**	15
FPC10	**10a**	62
FPC12	**10b**	48
FPC14	**10c**	30
FPC16	**10d**	11
FPC18	**10e**	4
FPLB	**10f**	30
FDACC[a]	**-**	35
Control	**18**	50

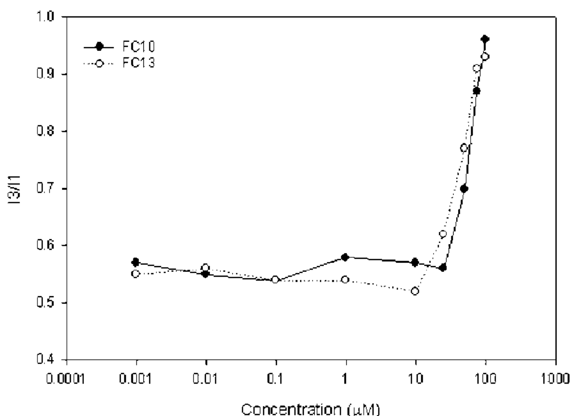


Fig. 8: Plot of I3/I1 ratio of the pyrene spectrum with respect to concentration of folate labeled amphiphile

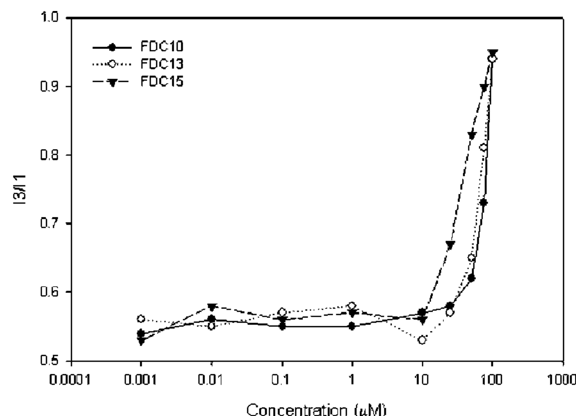


Fig. 9: Plot of I3/I1 ratio of the pyrene spectrum with respect to concentration of folate labeled amphiphile

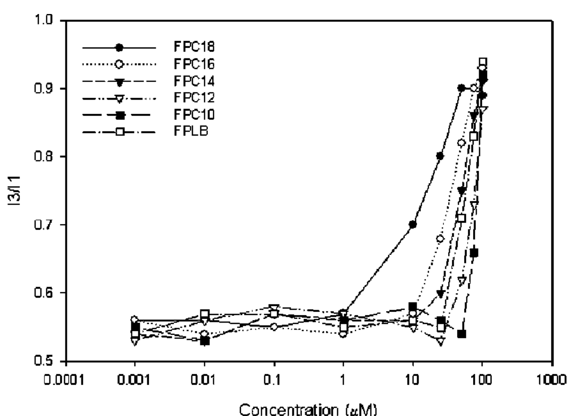


Fig. 10: Plot of I3/I1 ratio of the pyrene spectrum with respect to concentration of folate labeled amphiphile

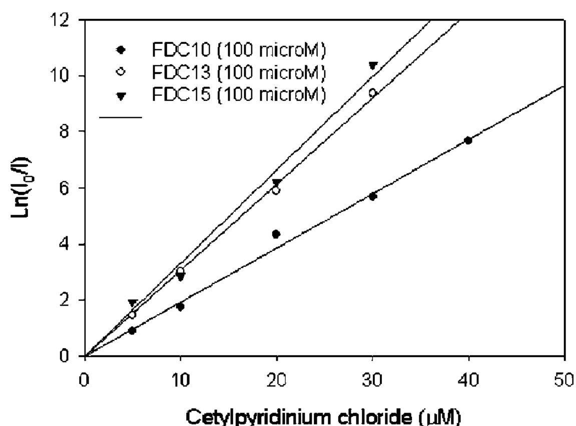


Fig. 11: Plot showing quenching of pyrene fluorescence with increasing concentration of cetylpyridinium chloride

Steady state fluorescence quenching of pyrene was used to measure aggregation number of micelles (86). In this method, it is assumed that the probe concentration is low when compared to micelles such that only one probe occupies a micelle and no emission takes place from micelles where both the probe and the quencher reside. Such a situation can be compared to distribution of *m* random objects in *n* boxes. Thus the distribution of the probe and the quencher among micelles follow Poisson statistics and the luminescence intensity of such a system is governed by

$$\frac{I}{I^0} = e^{-\left(\frac{[Q]}{[M]}\right)}$$

where I = Fluorescent intensity in presence of quencher

I^0 = Fluorescent intensity in the absence of quencher

[Q] = Concentration of quencher

[M] = Concentration of micelles

Now the term [M] can be written as

$$[M] = \frac{[C_{total}] - CMC}{n_{agg}}$$

where Ctotal=Concentration of the amphiphile in solution

cmc = Critical micellization concentration

nagg = Aggregation number

Thus aplot of in $\ln\left(\frac{I^0}{I}\right)$ against the quencher concentration [Q] yields straight line

with slope as $[M]^{-1}$ where the amphiphile $[C_{total}]$ and probe concentrations are contant. Aggregation number for the FDC(n) series of amphiphiles could be measured by using pyrene as the fluorescent probe and cetylpyridiniumchloride as a quencher (Fig 3). Fromthe queation above the aggregation number N_{agg} was calculated using the cmc(s) of the amphiphilic molecules, total concentration of the amphiphiles used and the micelle concentration (table 3).

Solubility of a model lipophilic drug, paclitaxel, was determined in the presence of FDC15 and FPC18 above their cmc(s). The aqueous solubility of paclitaxel was found to be 0.25µg/mL. FDC15 and FPC18 enhanced the solubility of paclitaxel by 85% and 62% respectively. Though the folate labeled amphiphiles did not increase the solubility to an extent that they can be considered as an alternative to Cremophor EL, but they can be used for the purpose of drug delivery in lieu of their targeting efficiency as cytotoxic activity elicited by a drug depends on its intracellular concentration.

Table 3 Aggregation number for FDC(n) series of amphiphiles

Amphiphile	Aggregation number [N_{agg}]
FDC10	9
FDC13	18
FDC15	28

Conclusion

The understanding of tumor biology has come a long way in terms of its cause, therapy and chemoprevention. But the question of specificity of antitumor agents towards diseased tissues still remains to be addressed. Targeted therapies based on hindering cell signaling pathways have evolved and are specific to tumor cells. But they are usually used in addition to the standard chemotherapeautic agents. The dose limiting toxicity results from the nonspecific cytotoxicity of these chemotherapeutic agents. Thus it is of utmost importance that these agents be delivered by targeted delivery minimizing dose limiting toxic side effects. In this manuscript, folated ligand conjugated amphiphilic molecules as micellar drug delivery systems were reviewed. The feasibility of this folate receptor based targeted delivery system approach that deploys micelles created by amphiphililc surfactants has been established. A great advantage of targeting with amphiphiles is its versatility because of the diverse array of targeting ligands that can be attached to amphiphilic. These amphiphilic molecules may range from small molecule

surfactants as reported here or may be large block copolymers such as pluronics that form polymeric micelles. Micelles from small molecule surfactants and amphiphiles are known to be unstable in biological systems due to extensive dilution in the body and interaction with plasma proteins. This can be overcome by the use of block copolymers which form stable polymeric micelles in biological systems.

The advent of these novel folate receptor based vitro methods coupled with deployment of block co polymers that are commercially available and have been in used in approved pharmaceutical products, give a compelling case for disciplined pre clinical evaluation for drug targeting in oncology space. Significant body of work needs to be completed, however, before such exciting opportunities can be advanced from academic laboratories into clinical evaluation to meet the unmet needs.

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Immunogenecity of a *Brucella abortus* S19 Glyco-conjugate Vaccine Consisting of Lipo-polysaccharide and Outer Membrane Protein in Cattle Calves

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Abstract

A glyco-conjugate vaccine consisting of lipo-polysaccharide (LPS) and the outer membrane protein (OMP) of *Brucella abortus* S19 strain was prepared. Cattle calves were inoculated with 50 µg of the glyco-conjugate vaccine. Separate group of calves was vaccinated with live, attenuated *B. abortus* S19 vaccine. The humoral immune response in calves was assessed by an indirect ELISA on days 21, 60, 90 and 120 post-vaccination. The glyco-conjugate vaccine was able to induce strong and comparable immune response against both components like the live, attenuated S19 vaccine. The IgG1 and IgG2 subtypes were prominent in the antibody response. In addition, the glyco-conjugate vaccine was able to induce a cell mediated immune response as indicated by the expression of IFNγ in a whole blood stimulation assay using inactivated whole bacterial antigen or OMP. In these aspects the glyco-conjugate vaccine was similar to the live, attenuated S19 vaccine. Results of the study indicate that the glyco-conjugate vaccine may be a useful vaccine for inducing potent immune responses in cattle.

Keywords: *Brucella abortus*, cattle, glyco-conjugate vaccine, lipo-polysaccharide, outer membrane protein

Introduction

Brucellosis is an economically important disease of the livestock industry in India. The disease caused by *Brucella abortus (B.abortus)* is manifested by endometritis, early embryonic mortality, infertility, abortion, retention of foetal membranes, increase in inter-calving period and loss of production in females. It also causes orchitis and decrease in semen quality in males. Brucellosis is controlled by vaccination using live attenuated *B.abortus* S19 (smooth) or RB51 (rough mutant). There are limitations with the live vaccines – only female cattle or buffalo between the age group of 4-12 months can be vaccinated while adult female or male animals cannot be included in the vaccination programme. Subunit vaccines have been considered as candidate vaccines against brucellosis. Winter *et al* (1) reported that a single vaccination with a porin and smooth lipopolysaccharide from *B.abortus* strain 2308 offered equivalent protection as achieved by vaccination with live attenuated strain 19. Mice immunized with a *Brucella* O-polysaccharide bovine serum albumin conjugate were protected against challenge with *Brucella melitensis* strain H38 (2). Other subunits of *B.abortus* like Ribosomal protein L7/L12 (3), outer membrane proteins (4), YajC and an 18 kDa lipoprotein (5, 6, 7) have also been reported to be immunogenic with some degree of protection.

We investigated the immunogenicity of a glyco-conjugate vaccine consisting of the *B.abortus* lipo-polysaccharide and OMP in cattle.

Materials and Methods

Cattle calves

Naïve unvaccinated crossbred male cattle calves aged 11-12 months, free of Brucella antibodies [tested using Rose Bengal Test (RBT) and the BRUCELISA Kit (VLA, UK)], were used in the present study for assessing the immune response to vaccination against Brucella.

Bacterial strains and growth

The *B.abortus* S19 (vaccine strain) used in this study was obtained from the Animal Disease Research Laboratory (ADRL), National Dairy Development Board (Anand, India) and was maintained according to propagation methods for *B.abortus* described by Alton *et al* (8). *B.abortus* S19 vaccine (Bruvax® B.No:01/08) produced by Indian Immunological Limited was used as positive control for assessing the immunogenecity of the glyco-conjugate vaccine in cattle calves. For preparation of the glyco-conjugate vaccine, *B.abortus* S19 strain was grown in an aerated stirred-tank bioreactor using soya casein digest medium (9)

Preparation of glyco-conjugate vaccine

Isolation of Lipopolysaccharide (LPS) from *B. abortus S19*

LPS from whole bacterial cells was extracted using the procedure described by Yi and Hackett (10). Briefly, LPS was extracted using 4M guanidine isothiocyanate and chloroform. The isolated LPS in the aqueous phase was pooled after three repeated extractions and analyzed for carbohydrate content using DuBoie's method (11). The purified LPS was electrophoresed on 12% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore Corporation, Bedford, MA) (12). The LPS fractions were detected using polyclonal rabbit anti-serum (1:2000) raised against *B.abortus* S19. Horseradish peroxidase labelled goat anti-rabbit IgG (Sigma, USA) at 1:1000 dilution was used as secondary antibody.

Extraction of Outer Membrane Protein (OMP) complex from *B.abortus* S19

Extraction and purification of OMP from physically disrupted, *Brucella* whole cell, was done using the method described by Verstreatre *et al* (13). Disruption was accomplished by two passages through a high-pressure cell disrupter at 40,000 lb/sq.inch (Constant Disruption Systems, UK). The disrupted suspension was centrifuged at 3,000 x g for 20 min at 4°C and the supernatant was centrifuged at 150,000 x g for 60 min at 4°C to pellet the crude membranes, which were resuspended at a concentration of 10 to 20 mg of protein per ml in Tris buffer. Detergent extraction of cytoplasmic membranes was performed using 0.01% Triton X-100 (Sigma, USA). The resultant insoluble material was dialyzed against Tris buffer at 4°C for 72 h with repeated changes. The OMP-rich fraction was subjected to digestion overnight at 37°C with egg white lysozyme (Sigma U.S.A) (1mg/50mg of protein). The OMP fraction was solubilized with equal volumes of buffer containing Triton X-100 and 50 mM EDTA and the samples were centrifuged at 100,000 x g for 20 min at 4°C, and the supernatants were held at 4°C. The supernatants were concentrated using tangential flow filtration using a 100 kDa cassette (Pall, India) followed by diafiltration using 10 mM Tris buffer (pH 7.5). Finally the materials were filtered through a 0.2 μ filter and stored at 4°C.

The purified OMP was electrophoresed on a 12% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore Corporation, Bedford, MA) (12). Detection of the antigenic components was done as described earlier for LPS.

Conjugation of Lipopolysaccharide with Outer Membrane Protein complex

LPS was chemically conjugated to OMP by the method of Beuvery *et al* (14) using 1-ethyl-3-(3-dimethylaminopropyl) carbo-di-imide (EDAC) (Sigma, USA). This mixture was analyzed on 12% SDS-PAGE. The LPS-OMP glyco-conjugate was used for preparing the vaccine with aluminium hydroxide gel as adjuvant. A single dose vaccine contained 50 µg of LPS and OMP glyco-conjugate.

Experimental Groups

Three groups consisting of six male cattle calves of 11-12 months of age were tested using RBT, BRUCELISA Kit (Veterinary laboratory agencies, UK) and an in-house developed c-ELISA for seronegativity for brucella antibodies. The animals were administered with 50 µg of the glyco-conjugate vaccine prepared from *B.abortus* S19. The animals were boosted with another dose of the glyco-conjugate vaccine on day 90 post-vaccination. Blood samples were collected on 0, 21, 60, 90 and 120 days post-vaccination for estimation of antibodies against LPS and OMP. Heparinized blood samples were collected on the same days for whole blood IFNγ assay.

Antibody response by ELISA

The sera separated from the clotted blood were used to estimate the serum antibody titre against Brucella antigens OMP and LPS and also the bovine antibody isotypes using an indirect ELISA (15). The optimal concentration of antigen and dilution of the serum were determined by performing a checker board titration of purified LPS and OMP fraction and known positive and known negative cattle serum. ELISA plates (Nunc Maxisorp™,The Netherlands) were coated with 100 ng of LPS and OMP in carbonate-bicarbonate coating buffer (pH 9.0). After 1 hour of incubation at 37ºC the plates were washed with phosphate buffered saline containing 0.05% Tween 20 (PBST). The wells were blocked with 3% Skim Milk Powder in PBST (S-PBST). Test sera were incubated after pre-dilution with S-PBST (1 in 50), serially diluted and incubated at 37º C for 1 h. The plates were washed with PBST and 100 µl of HRP conjugated anti-bovine IgG at appropriate dilution in S-PBST was added to the plates. After incubation at 37º C for 1 h and the plates were washed with PBST and 100µl of Chromogen / Substrate mix (TMB/ Hydrogen Peroxide (Sigma, USA) was added to the plates. The plates were incubated in dark for 10 min at room temperature. The reaction was stopped with 1 M Sulfuric acid (Emerck, Germany) and the plates were read at 450 nm using ELISA plate reader (Multiscan®Titertek™, Finland). The log reciprocal of the dilution showing optical density value close to the cut-off was taken as the serum antibody titre.

The presence of antigen specific serum IgG1 and IgG2, were determined using a sheep anti bovine IgG1 and IgG2 HRP (AbD Serotec, UK). The end point titres for different isotypes were determined as in the case of antibody response described above.

Interferon gamma assay

The whole blood IFNγ production assay was performed as per the procedure described elsewhere (16) to assess the cell mediated immune response. The first step consisted of a short-term culture of heparinised whole blood in the presence or absence of purified Brucella OMP,

killed whole bacteria and the second step was a capture ELISA for the measurement of IFNγ levels in the plasma of the induced blood. The assay was set up within 24 h of blood collection and heparinised blood samples were stored at room temperature up to the time of setting up the assay, consistent with the BOVIGAM® protocol. Antigen preparations of *B.abortus* S19 were used to stimulate the cells in whole blood. For this purpose, 10 µg/ml of the purified OMP or the killed bacterium of *B.abortus* S-19 was used. Poke Weed Mitogen (PWM) (10 µg/ml) was used in duplicate as positive control to demonstrate viable cells capable of producing IFNγ were present in each blood sample. Finally, duplicates of 250 µl of whole blood were processed without induction to obtain baseline plasma samples. As further specificity controls, blood samples collected from the unvaccinated naïve control animals were stimulated with the above antigens as above. The 24 well plates were incubated for 24 h at 37°C in a CO2 incubator. Then the plates were centrifuged at 1000 rpm for 10 min at 4°C. A 100 µl volume of supernatant was pipette out from each well into a new 96 well plate and stored at -20°C until required for testing by ELISA. Bovine IFNγ specific antibody pairs suitable for use as a sandwich ELISA were obtained from AbD Serotec (UK). ELISA was performed with suitable standards. Known amounts of IFNγ were tested in duplicate in the first two columns of each ELISA plate and the quantity of IFNγ in each unknown sample was estimated from the standard curve obtained from the known standards.

Statistical analysis

All the data were analysed statistically for their significance using standard procedures described by Snedecor and Cochran (17).

Results and Discussion

Brucellosis is an economically important disease of the dairy industry. Unlike in developed countries, the 'test and slaughter' method is not practiced for the control and eradication of the disease in India. The disease is controlled by the use of live, attenuated vaccines. Availability of safe and efficacious vaccines will improve vaccine coverage and also the use of vaccination as a primary means of disease control. The live, attenuated vaccines have certain limitations and several research groups are working on sub unit vaccines and plasmid DNA vaccines against brucellosis. These vaccines can be used in both sexes and all age groups.

Isolation of *B.abortus* LPS, OMP and preparation of LPS-OMP glyco-conjugate

From 1g of wet cells 500 µg of LPS and 700 µg of OMP could be extracted. LPS appeared as a wide smear of high molecular weight (100-200 kDa) fraction and 2-4 bands of low molecular weight fraction (30-80kDa).(data not shown) OMPs were separated as three groups of proteins, namely, group 1 (94 or 88 kDa), group 2 (36–38 kDa), and group 3 (31–34 and 25–27 kDa). The molecular weight of the glyco-conjugate was analyzed on SDS-PAGE and was higher than LPS. The bands corresponding to OMP fractions were absent indicating successful conjugation. Further, the increase in estimated molecular weight of the glyco-conjugate on SDS-PAGE compared to LPS or OMP indicated conjugation of the molecules (data not shown). The analysis of the LPS-OMP glyco-conjugate showed a polysaccharide to protein ratio of 3:1

Antibody response by ELISA

Cattle calves were inoculated once with glyco-conjugate vaccine containing 50 µg of LPS+OMP conjugate. The glyco-conjugate

vaccine was able to elicit a strong antibody response in cattle similar to that of the live, attenuated vaccine as measured by a specific indirect ELISA (Table 1a & 1b). The titres were significantly higher and comparable with the S19 vaccine. The animals vaccinated with LPS-OMP glyco-conjugate vaccine showed a strong IgG antibody response up to day 120 post-vaccination comparable to the S-19 vaccinated groups (P<0.05). Following booster vaccination on day 90 there was a strong anamnestic response in the glyco-conjugate vaccine group. As per the manufacturer's instructions the S-19 vaccine group were not revaccinated.

Table 1a: Mean antibody titres against purified lipo-polysaccharide of Brucella abortus S-19 in i-ELISA in cattle calves vaccinated with Brucella abortus S-19 glyco-conjugate vaccine and controls. († - the vaccinated groups did not differ significantly in the antibody response (P>0.05) whereas there as a highly significant difference when compared with the unvaccinated controls (P<0.01).

Vaccine groups	0 dpv	21 dpv†	60 dpv†	90 dpv†	120 dpv†
Glyco-conjugate vaccine	1.76±0.13	2.42±0.46	2.30±0.21	2.96±0.25	3.20±0.13
S-19 vaccine	1.76±0.-13	2.84±0.3	2.36±0.39	2.90±0.30	3.20±0.16
Unvaccinated control	1.70	1.70	1.82±0.16	1.70	1.70

Table 1b: Mean antibody titres against purified outer membrane protein complex of Brucella abortus S-19 in i-ELISA in cattle calves vaccinated with Brucella abortus S-19 glyco-conjugate vaccine and controls. († - the vaccinated groups did not differ significantly in the antibody response (P>0.05) whereas there as a highly significant difference when compared with the unvaccinated controls (P<0.01).

Vaccine groups	0 dpv	21 dpv†	60 dpv†	90 dpv†	120 dpv†
Glyco-conjugate vaccine	1.76±0.13	2.90±0.25	3.08±0.27	3.62±0.63	3.87±0.53
S-19 vaccine	1.76±0.-13	3.20±0.16	3.20	3.87±0.78	3.20±0.46
Unvaccinated control	1.70	1.70	1.76±0.-13	1.70	1.70

The isotypes of the specific antibodies were IgG1 and IgG2 for the live attenuated vaccine and the LPS-OMP glyco-conjugate vaccine groups (Table 2a & 2b). The *B.abortus* LPS is known to persist on the surface of antigen presenting cells and thus may induce a prolonged antibody response (18). In this study, the antibody levels remained high until day 60 post vaccination and an anamnestic response was noticed after booster on days 90 and 120 post-vaccination in animals LPS-OMP glyco-conjugate vaccine group. Booster vaccination is not recommended for the live attenuated vaccine. The isotype (IgG) and subtype (IgG1 and IgG2) responses induced by the LPS-OMP glyco-conjugate vaccine may also indicate a Th1 and Th2 type response induced by the vaccine (19).

Table 2a: Isotype specific immune response against purified lipo-polysaccharide of Brucella abortus S-19 in i-ELISA in cattle calves vaccinated with Brucella abortus S-19 glyco-conjugate vaccine and controls. Numbers indicate mean antibody titers. († - the vaccinated groups did not differ significantly in the antibody response (P>0.05) whereas there as a highly significant difference when compared with the unvaccinated controls (P<0.01).

Vaccine groups	IgG1					IgG2				
	0 dpv	21 dpv†	60 dpv†	90 dpv†	120 dpv†	0 dpv	21 dpv	60 dpv†	90 dpv†	120 dpv†
Glyco-conjugate vaccine	1.40	2.60±0.30	2.06±0.39	2.06±0.54	3.45±0.40	1.46±0.13	1.40	1.58±0.27	1.58±0.27	2.84±1.75
S-19 vaccine	1.52±0.16	2.42±0.34	2.30±0.56	2.30±0.52	2.60	1.40	2.12±0.16	1.82±0.34	1.88±0.27	2.36±0.13
Unvaccinated control	1.40	1.40	1.46±0.13	1.40	1.52±0.16	1.40	1.40	1.40	1.40	1.52±0.16

Table 2b: Isotype specific immune response against purified outer membrane protein complex of Brucella abortus S-19 in i-ELISA in cattle calves vaccinated with Brucella abortus S-19 glyco-conjugate vaccine and controls. Numbers indicate mean titers. († - the vaccinated groups did not differ significantly in the antibody response (P>0.05) whereas there as a highly significant difference when compared with the unvaccinated controls (P<0.01).

Vaccine groups	IgG1					IgG2				
	0 dpv	21 dpv†	60 dpv†	90 dpv†	120 dpv†	0 dpv	21 dpv†	60 dpv†	90 dpv†	120 dpv†
Glyco-conjugate vaccine	1.40	2.60±0.30	2.30±0.64	1.94±0.39	3.69±0.78	1.40	1.58±0.40	1.64±0.13	1.64±0.13	2.54±0.65
S-19 vaccine	1.40	2.54±0.33	2.60±0.37	2.48±0.16	2.48±0.16	1.40	1.58±0.27	2.12±0.45	1.94±0.13	1.70±0.21
Unvaccinated control	1.40	1.40	1.52±	1.40	1.46±0.13	1.40	1.40	1.52±0.27	1.40	1.40

Interferon gamma assay

The LPS-OMP glyco-conjugate vaccine was able to induce an OMP specific and whole bacterial cell specific cell mediated immune response as shown by the IFNγ expression in LPS-OMP glyco-conjugate vaccine group (Table 3). The IFNγ response is important in activating macrophages and killing of intra-cellular *brucellae* (20). The IFNγ expression in response to whole cell antigen in S19 vaccinated calves and the LPS-OMP glyco-conjugate vaccinated calves was comparable. These results suggest that the OMP component of the LPS-OMP glyco-conjugate vaccine may induce a specific CMI response in vaccinated cattle. *B.abortus* LPS is 10,000 fold less pyrogenic than *E.coli* LPS (21) and is a potent stimulator of antigen presenting cells. In the present study a glyco-conjugate vaccine was prepared using the LPS and OMP of *B.abortus* and the vaccine was tested for its protective efficacy against challenge with wild type *B. abortus* (data not shown). Other researchers have reported the use of *B. melitensis* LPS (20), *B.melitensis* LPS covalently conjugated to BSA (4) and *B. melitensis* LPS non-covalently conjugated to *Neisseria meningitidis* OMP (15). To our knowledge this is the first report of using *B.abortus* LPS and OMP in a chemically conjugated form for the preparation of a vaccine

The results presented here showed that a glyco-conjugate vaccine could induce both Th1 and Th2 cells marked by a strong antibody response and IFNγ response in cattle. Future studies will determine the duration of immune response in cattle and the efficacy of the vaccine in protecting cattle from *brucella* infection.

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Table 3: Interferon gamma response as a measure of cell mediated immune response in cattle calves vaccinated with Brucella abortus S-19 glyco-conjugate vaccine and controls against purified outer membrane protein complex (OMP) of Brucella abortus S-19 and acetone killed whole cell antigen (WCA) from stimulated bovine lymphocytes. Numbers indicate mean quantity of interferon gamma secreted after stimulation (pg/ml).

Vaccine groups	OMP					WCA				
	0 dpv	21 dpv	60 dpv	90 dpv	120 dpv	0 dpv	21 dpv	60 dpv	90 dpv	120 dpv
Glyco-conjugate vaccine	4.43±9.02	8.37±5.05	134.62±124.75	135.76±166.25	6.85±1.13	1.24±21.70	54.90±79.30	45.05±19.36	90.21±126.95	101.10±6.19
S-19 vaccine	4.94±6.65	16.98±13.87	143.39±102.61	142.58±65.55	4.65±2.29	3.89±2.90	94.91±127.69	39.89±19.71	97.80±113.03	95.22±19.15
Unvaccinated control	7.54±3.01	0.70±5.09	11.73±5.04	3.16±1.80	2.77±1.68	3.04±8.78	4.00±5.32	6.29±4.26	4.66±7.07	1.84±1.91

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Biochemical Studies on the Effect of Volatile Oil of *Thymus capitatus* in Alloxan-Induced Diabetic Rats

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Abstract

Administration of thyme oil @ 0.1 mL kg^{-1} body weight orally once daily for 28 days to diabetic rats revealed progressively declined values of serum glucose from 483.30±1.35 mg% on day 0-113.20±1.00 mg% at the end of the trial. Serum total cholesterol levels revealed progressively inclining values till 21 days and thereafter declining to 107.40±1.20 mg% at the end of the trial. However, serum HDL, triglycerides, serum urea nitrogen and creatinine levels remained within normal limits as recorded in glibenclamide treated diabetic rats except a mild increase in ALT and AST levels was recorded on day 7. The histological findings revealed regenerative changes in the pancreas, liver and kidney. The present results demonstrated the antihyperglycemic and antilipidemic effects of thyme oil.

Key words : Antihyperglycemic, thyme oil, biochemical changes, alloxan induced diabetic rats)

Diabetes mellitus, one of the most common metabolic disorders affecting nearly 10% of world population has a significant impact on health, quality of life and life expectancy of patients as well as on the health care system. Its incidence is increasing rapidly at global level, particularly in developing countries. Despite the presence of known antidiabetic medicines in the pharmaceutical market, diabetes and the related complications continue to be a major health problem. On the other hand, traditional medicinal plants have been used since the ancient times by physicians and laymen to treat diabetes and its related complications, presenting a stirring prospect for the expansion of an alternative way of treatment of this disease. Herbal drugs are prescribed widely, even when their biologically active compounds are unknown, because of their effectiveness, lesser side-effects and relatively low cost. There are more than 1200 species of medicinal plants recognized throughout the world for their ability to treat diabetes mellitus. To date only a few of the medicinal plants grown in Africa have been used in folk medicine the treatment of diabetes mellitus (22,35). The whole plant as such or its various parts namely seeds, fruits, flowers, leaves, bark, stem, roots, bulbs, sprouts, corms and immature pods have been commonly employed in folk medicine to treat diabetes mellitus.

Of various plants grown in Libya, *Thymus capitaus*, a perennial aromatic sub-shrub native to the western Mediterranean area has been proven widely to possess various medicinal

properties including antimicrobial, antifungal, antioxidant, antitumorogenic, antimutagenic and anti-inflammatory activities (9,20,25). However, no studies have been reported on the antidiabetic effect of this plant.

Therefore the present study was carried out to assess the antidiabetic and related effects of volatile oil extracted from *T. capitatus* in alloxan-induced diabetic rats.

Materials and Methods

Plant material and extraction of volatile oil: The fresh leaves of *T. capitatus* were collected from the areas of south of El Beida to Laruloda, Libya. The authenticity of the plant species was identified by scientists in the Department of Botany, Faculty of Science, Al Fateh University, Tripoli, Libya. The volatile oil was extracted by hydrodistillation method as per the procedure described by Balbaa *et al*.(8).

Chemicals and drugs: Alloxan monohydrate was purchased from Sigma Chemicals (St. Louis, USA). All the biochemical kits used in this experiment were obtained from Bicon diagnostik, Germany and all the other chemicals used were of analytical grade.

Animals: Male albino rats weighing 100-150 g (bred in the Animal House, Omar Al Mukhtar University, Al Beida, Libya) were used in the present experiment. The animals were housed in polypropylene cages, fed on a standard pellet diet and water given *ad libitum*. All the studies were conducted in accordance with the NRC (24).

Experimental induction of diabetes: Animals were deprived of feed for 24 h but were allowed free access to water before administration of alloxan. Alloxan monohydrate was dissolved in sterile normal saline and administered @150 mg kg^{-1} body weight intraperitoneally as a single dose (6). The rats found hyperglycemic after 48 h of alloxan administration, with blood glucose levels above 250 mg dL^{-1} were used for further studies.

Design of the experiment: A total number of 60 rats were used and they were divided into 6 groups of 10 rats each. Non-diabetic rats were used for the group I and II while diabetic rats were used for the remaining groups. Group I served as non-diabetic control which received no treatment while group II, consisted of non-diabetic rats, received volatile oil of thyme @ 0.1 mL kg^{-1} body weight. Group III consisted of diabetic rats which received corn oil @ 0.1mL kg^{-1} body weight and group IV consisted of diabetic rats treated with volatile oil of thyme @ 0.1 mL kg^{-1} body weight. Group V served as drug control (diabetic rats treated with glibenclamide @ 5 mg kg^{-1} body weight) while group VI served as diabetic control which received no treatment.

Administration of volatile oil and Glibenclamide: The volatile oil of thyme was dissolved in corn oil while glibenclamide was dissolved in distilled water and administered orally using a feeding needle. All doses were administered orally once daily for a period of 28 days.

Collection of blood samples: Blood samples were collected at 0, 7, 14, 21 and 28 days of the trial from the orbital sinus using capillary tubes after partly anaesthetizing the rats.

Parameters studied

Biochemical estimations: Serum glucose was determined by GOD-POD method (32), total cholesterol was determined by CHOD-PAP method (3) and triglycerides concentration was determined by GPO-PAP method[15]. Serum HDL and LDL concentrations were determined by the method described by Rifai and Warnick (27). Serum urea nitrogen was determined by diacetyl monoxine method[33] and creatinine was determined by alkaline picrate method (31). Alanine amino transferase (ALT)

and Aspartate amino Transferase (AST) activities in the serum were determined by Reitman and Frankel (26) method.

Pathological studies: One rat from each group was sacrificed at 0, 7, 14, 21 and 28 days. The organs namely liver, kidney and pancreas were examined for any gross abnormalities and preserved in 10% formalin, processed by routine paraffin embedding method and stained by haematoxylin and eosin for histopathological examination.

Statistical analysis: The mean and standard error for all the groups was calculated. The mean values were compared with using students' t-test at 5% level of significance.

Results and Discussion

The yield of volatile oil obtained from the fresh leaves of *T. capitatus* was found to be 2.49%. The present findings were in accordance with that of Alonso (4) who reported a yield of 0.8-2.5% of volatile oil extracted from the fresh leaves and twigs of *T. capitatus*.

The biochemical findings recorded in both treated as well as untreated rats are shown in Table 1 and 2 and Fig. 1a-d. The untreated diabetic control rats showed significant ($p<0.05$) progressively increased values of serum glucose, cholesterol, LDL, triglycerides, ALT, AST, serum urea nitrogen and creatinine and decreased values of serum HDL towards the end of the trial. The biochemical findings correlated well with that of histological findings which revealed progressive necrosis of the islet cells of the pancreas (Fig. 1) and degenerative changes in the renal tubules and hepatocytes.

The diabetic rats treated with volatile oil of thyme recorded significant ($p<0.05$) decreasing values of serum glucose towards normal at the end of the trial, showing a mean value of 483.32±1.35 mg% and 113.20±1.00 mg% on day 0 and day 28, respectively. The serum cholesterol levels showed progressively inclining values from 82.63±0.93 mg% at 0 day to 144.00±1.83 mg% at 14 days and thereafter declining to 107.40±1.20 mg% at the end of the trial. However, serum HDL and triglycerides remained within normal limits throughout the entire period of study while LDL levels started increasing until day 14 (30.70±1.12 and 89.40±0.79 mg% at 0 and 14 days, respectively) and

Table 1: Serum glucose and triglyceride levels* in normal, diabetic and thyme oil treated rats

	Glucose (day)					Triglyceride (day)				
Group	0	7	14	21	28	0	7	14	21	28
1	84.38^{a} ±0.68	84.60^{a}±0.75	85.25^{a}±0.61	85.53^{a}±0.67	85.10^{a}±0.90	83.38^{a}±0.83	84.28^{a}±0.83	85.20^{a}±0.58	85.60^{a}±1.67	82.20^{ac}±2.00
2	83.17^{a}±1.36	81.76^{a}±0.75	84.20^{a}±1.41	85.47^{a}±0.64	85.20^{a}±1.00	83.20^{a}±0.90	83.88^{a}±1.16	85.20^{a}±0.58	84.27^{a}±1.10	84.30^{a}±2.10
3	484.40^{b}±1.09	444.60^{b}±24.66	503.00^{b}±7.77	507.33^{b}±8.19	537.00^{b}±5.00	83.27^{a}±1.19	83.72^{a}±1.47	95.00^{b}±1.29	156.00^{b}±7.57	211.00^{a}b±1.00
4	483.32^{b}±1.35	257.76^{c}±7.14	141.50^{c}±0.96	120.67^{c}±0.67	113.20^{c}±1.00	81.83^{a}±0.70	85.36^{a}±0.47	82.90^{a}±1.26	85.07^{a}±0.68	85.20^{a}±1.00
5	483.33^{b}±0.79	116.92^{e}±2.37	92.00^{e}±2.83	93.33^{d}±2.40	88.00^{a}±2.00	82.93^{a}±0.82	84.20^{a}±1.10	85.15^{a}±0.61	84.80^{a}±0.70	80.30^{c}±0.10
6	482.30^{b}±1.04	537.92^{f}±11.16	527.50^{f}±7.50	526.00^{e}±3.06	541.30^{b}±1.10	84.27^{a}±0.74	84.96^{a}±0.47	96.00^{b}±0.82	159.33^{b}±4.67	210.00^{b}±2.00

*: Values expressed in mg% **: Values with different superscripts differ significantly

Table 2: Cholesterol, HDL and LDL levels* in normal, diabetic and thyme oil treated rats

	Cholesterol (day)					HDL (day)				
Group	0	7	14	21	28	0	7	14	21	28
1	$84.40^{a}\pm0.57$	$84.64^{a}\pm0.72$	$84.70^{a}\pm0.52$	$86.13^{a}\pm1.27$	$85.20^{a}\pm1.00$	$35.73^{a}\pm1.09$	$34.64^{a}\pm1.61$	$33.05^{a}\pm0.61$	$32.37^{a}\pm0.98$	$31.30^{a}\pm0.70$
2	$84.20^{a}\pm0.65$	$84.00^{a}\pm0.67$	$85.20^{a}\pm0.58$	$85.60^{a}\pm0.70$	$85.10^{a}\pm1.10$	$33.53^{b}\pm0.96$	$34.56^{a}\pm1.09$	$33.75^{a}\pm0.97$	$34.50^{b}\pm1.15$	$31.00^{a}\pm1.00$
3	$81.43^{a}\pm0.46$	$83.36^{a}\pm0.77$	$129.50^{b}\pm0.96$	$145.33^{b}\pm1.76$	$178.00^{b}\pm10.00$	$36.00^{ac}\pm0.57$	$34.04^{a}\pm0.89$	$34.10^{a}\pm0.78$	$25.33^{c}\pm1.76$	$17.00^{c}\pm1.00$
4	$82.63^{a}\pm0.93$	$85.52^{a}\pm0.55$	$144.00^{c}\pm1.83$	$123.33^{c}\pm1.33$	$107.40^{c}\pm1.20$	$35.57^{a}\pm1.23$	$34.64^{a}\pm0.40$	$37.65^{b}\pm0.98$	$34.47^{b}\pm1.10$	$31.50^{a}\pm0.90$
5	$84.47^{a}\pm0.62$	$84.72^{a}\pm0.35$	$85.25^{a}\pm0.50$	$92.67^{e}\pm1.76$	$85.10^{a}\pm0.90$	$36.70^{ac}\pm0.58$	$34.28^{a}\pm0.84$	$31.00^{c}\pm1.29$	$32.93^{a}\pm1.83$	$32.70^{b}\pm0.10$
6	$83.07^{a}\pm0.91$	$84.80^{a}\pm1.02$	$134.85^{b}\pm1.58$	$156.00^{f}\pm4.16$	$183.00^{b}\pm15.00$	$37.23^{c}\pm0.46$	$35.52^{a}\pm0.97$	$31.25^{c}\pm0.60$	$28.00^{d}\pm1.15$	$18.00^{d}\pm0.00$

*: Values expressed in gm% **: Values with different superscripts differ significantly

thereafter started declining, reaching 58.46±0.94 mg% at the end of the trial. The hypoglycemic findings correlated well with histological findings which revealed regenerative changes in the islet cells of the pancreas (Fig. 2) towards the end of the trial.

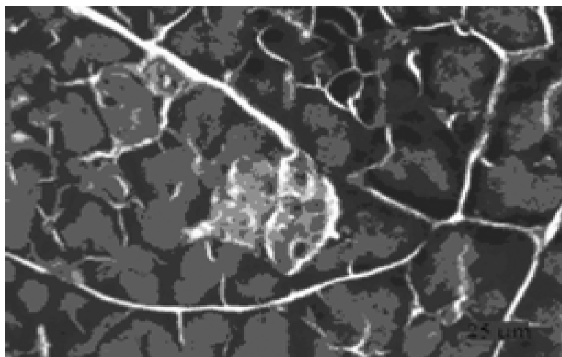

Fig. 1: Showing pancreatic islet cell necrosis-untreated diabetic rat (7 days)

A mild increase in serum ALT and AST in diabetic rats treated with volatile oil of thyme was recorded on day 7 (44.00±1.67 and 42.40±1.46 U L^{-1}, respectively). These enzymes started declining thereafter until the end of the trial to a value of 37.00±1.00 and 21.00±1.00 IU L^{-1} for ALT and AST, respectively. However, serum urea nitrogen and creatinine levels remained within normal limits until the end of the trial. These biochemical findings correlated with the histological findings which revealed no abnormal changes in the liver and kidney throughout the entire period of the study except for mild degenerative changes in the liver recorded on day 7.

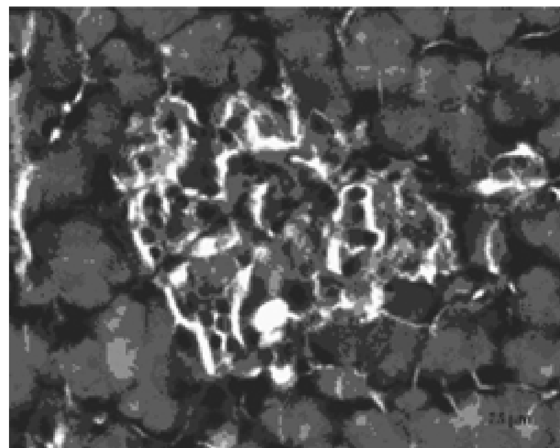

Fig. 2: Showing pancreatic islet cell regeneration-Thyme treated diabetic rat (21 days)

The present hyperglycemic findings observed in diabetic rats might be attributed to cause of destruction of beta cells of the pancreas by alloxan[34] as confirmed histologically. Abnormal lipid findings could be due to altered

lipid metabolism consequent to insulin deficiency. In diabetes mellitus, the utilization of impaired carbohydrate leads to accelerated lipolysis resulting in dyslipidemia as a result of insulin deficiency which fails to activate the enzyme lipoprotein lipase and hydrolyse the triglycerides (18). Increased levels of serum ALT and AST levels and decreased values of total protein and albumin might be as a result of hepatic damage (16,18,21). Increased serum urea nitrogen and creatinine concentrations might be due to renal insufficiency that is commonly encountered in uncontrolled diabetes mellitus (12).

The present hypoglycemic findings recorded in thyme-treated diabetic rats were also observed in observed in diabetic rabbits treated with volatile oil of *Nigella sativa* seeds after 4 and 6 h of treatment and *Myrtus communis* @ 50 mg kg^{-1} body weight once daily for a period of 1 week (1,28,29). Similar observations were also recorded in diabetic rats treated with black caravay (*Carum carvi* L.) oil for a period of 10 weeks (14).

The present antilipidemic findings observed in thyme-treated diabetic rats was also reported in healthy individuals and patients with coronary artery disease treated with garlic oil (11) and in normal healthy humans who received garlic essential oil @ 18 mg day^{-1} for 4 weeks (10). Similar observations were also observed in rats treated with *Nigella sativa* oil @ 800 mg kg^{-1} body weight for 4 weeks and in hypercholesterolemic patients @ 2.5 mL twice daily for 4 weeks (5,13).

The antihyperglycemic activity of thyme might be due to the presence of active principles similar to that of oral hypoglycemic agents which might act by stimulation of the beta cells of the pancreas to release insulin as evidenced histologically. *In vitro* and *in vivo* studies revealed that rosemarinic acid and luteolin inhibit the activities of enzymes, alpha glucosidase and alpha amylase and thus preventing the absorption of glucose in the small intestine (17,19,23) (Kim *et al*., 2000). The presence of such active compounds in *T. capitatus* might be responsible for the antihyperglycemic activity.

The antilipidemic response of *T. capitatus* might be due to the restoration of normal lipid metabolism consequent to the antihyperglycemic mechanism. Taku *et al*. (30) opined that thymol and carvacol significantly decrease serum cholesterol levels by increasing the microsomal geranyl pyrophosphate pyrophosphatase activity. The structural diversity of the isopropanoids which suppress cholesterol synthesis may be reconciled by their ability to increase pyrophosphatase activity, thus leading to the production of the endogenous, post-transcriptional regulator of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. Thymoquinone, a derivative of thymol has been reported widely to possess antilipidemic property (2,7). The presence of such compounds in *T. capitatus* might contribute to their antilipidemic property.

The normal biochemical and histological findings of the liver and kidney in thyme-treated rats might be as a result of restoration of normal functions secondary to antihyperglycemic effects of active compounds present in thyme.

Conclusion

The present study revealed the antihyperglycemic and antilipidemic effects of volatile oil of *T. capitatus.* However, the exact mechanism and the active compounds involved in hypoglycemic as well as hypolipidemic activities remains to be elucidated. In addition, the present findings also revealed the non-toxic effects of thyme oil when administered @ 0.1mL kg^{-1} body weight for 28 days as evidenced biochemically and histologically.

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Assessment of Rice Genotypes for Brown Plant Hopper Resistance Using Microsatellite Markers

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Abstract

Phenotypic response of the 16 rice genotypes to brown plant hoppers (BPH) was evaluated at flowering stage under field conditions. The results indicated that genotypes were classified into five groups from score 1 to score 9. A total of 88 alleles were detected by 37 polymorphic markers with an average of 2.17. Polymorphic information content (PIC) value varied from 0.92 for RM 483 to 0.98 for RM 84, with an average of 0.95. An efficient separation of 16 rice genotypes based on SSR data into three groups was achieved by using unweighted pair group method with arithmetic means (UPGMA) clustering procedure based on genetic similarity expressed by the Jaccard similarity coefficient (JSC). Genotypes that are derivatives of genetically similar type clustered more together. The results also indicated that land races which are potential sources for biotic stress exhibited wide range of reaction from score 1 (slight drying) to score 9 (all plants apparently dead) to brown plant hopper screening and therefore very useful for rice breeding programs, especially for genetic mapping studies and eventually for application of marker-assisted selection (MAS). From this study it was concluded that mapping population could be developed by using PTB 33 and BM 71 as donors to introgress genes for BPH resistance into cultivated high yielding rice cultivars

Key words: Brown plant hopper, Genetic diversity, Rice genotypes, SSR markers, variation

Introduction

Rice is a staple food for one-third of the worlds population and is grown on more than 148 million ha in wide range of ecosystems under varying temperature and water regimes. Most of the Worlds rice is cultivated and consumed in Asia, which constitutes more than half of the global population. Many biotic and abiotic stresses are continuing threat to rice productivity and sustainability. The major challenge is to overcome these constraints and produce high yielding rice varieties with multiple resistances to biotic and abiotic stresses possessing improved grain quality and nutritive value. Among the biotic stress, the brown plant hopper (BPH) *Nilaparvata lugens* Stal. is a serious insect pest of rice in Asia, causing severe yield losses in rice growing areas. It damages the crop by direct feeding and indirectly by acting as a vector for transmitting rice grassy stunt virus (RGSV) and yellow dwarf viral diseases. Under severe infestation, it causes complete death of crop popularly known as "hopper-burn" causing 100 % yield loss and this emerged as the major pest in tropical Asia during

green revolution of the 1966. Chemical control is often expensive, health hazardous, pollutes the environment and destroys the natural balance of BPH predators that help keep the BPH population in check, and can ultimately cause development of new, insecticide resistant strains. Therefore, the most economical and efficient method to control BPH is through host plant resistance as part of IPM (Integrated pest management) and developing resistant rice cultivars.

DNA based molecular markers have been used extensively to assess the genetic diversity of most crop species. Due to high efficiency, reproducibility, easy-to-use, co-dominance nature and high degree of polymorphism, microsatellite markers or simple sequence repeats (SSRs) are widely-used as molecular markers for fingerprinting germplasm to assess genetic diversity, pedigree analysis, evolutionary studies and genome mapping (6,8,19). Rice microsatellites have been demonstrated to be polymorphic between (2,4) and within rice populations (12). The unveiling of the rice genome draft sequence in public domain has given a vast choice of SSR markers (18,828) throughout the whole genome (7).

Major QTLs conferring resistance to BPH biotypes 1 and 2 have been reported (1,16,18). Two large effect QTLS namely *Qbp1* and Q*bp2* conferring resistance to BPH biotypes of China have been mapped to long arm of chromosome 3 and short arm of chromosome 4 respectively (14,10). Many donors possessing resistance have been identified and their resistance genes have been incorporated in the improved varieties. To date, 19 major genes have been identified (*Bph1 to Bph19)* so far in *indica* cultivars and wild species of *O. australiensis, O. eichingeri and O. officinalis* (23). A set of 15 genes has been assigned to rice chromosomes using molecular markers excepting *Bph 5, Bph 6, Bph 7 and Bph 8* (23). However many of the molecular markers reported so far are not tightly linked to the gene of interest and difficult to use them for molecular assisted selection. The Biotype 4 which is major occurrence in South Asian countries of India, Bangladesh and Srilanka and in a way towards developing resistant varieties for this biotype of BPH the present study was taken up with a goal to identify the genetic diversity among different genotypes coupled with the resistance to BPH.

Materials and Methods

Plant material and Screening for BPH Resistance

The experimental material consists of 16 genotype viz, Sambamasuri , Prabhat , Swarna , Improved Sambamasuri , Dhanya Lakshmi Deepthi , MO1, BM-71, PTB-33, PTB-18, PTB-21, PTB-22, PTB-25, PTB-28, PTB-31 and PTB-1. These cultivars were obtained from the Andhra Pradesh Rice Research Institute and Regional Agricultural Research Station (APRRI & RARS) Maruteru .The genotypes having varied response to brown plant hopper stress ranging from landraces to improved lines (Table 1). Field screening for evaluation of responses of genotypes to brown plant hopper stress at flowering stage was followed .The genotypes were grouped under 1,3,5,7 and 9 score categories on the basis of extent of crop damage as described in standard evaluation system for rice . IRRI (8).

Table 1. Scoring of rice genotypes for brown plant hopper resistance at flowering stage

Genotype	Parents	Source	**BPH SCORE**
Sambamasuri	TN (1) / Mahsuri /GEB-24	Bapatla	9
Prabath	IR 8 / MTU 3	Maruteru	9
Swarna	Vasista / Mahsuri	Maruteru	9
Improved Sambamasuri	TN (1) / Mahsuri /GEB-24	(DRR) Hyderabad	9
Dhanya Lakshmi	W12708/Sabermathi	Bapatla	7
Deepthi	Sowbhagya / ARC 6650	Maruteru	5
BM 71	IJ derivative	Maruteru	1
MO 1	Pure selection from local land races of Pattambi	Pattambi	5
PTB-33	Pure selection from local land races of Pattambi	Pattambi	1
PTB-18	-do-	Pattambi	3
PTB-21	-do-	Pattambi	9
PTB-22	-do-	Pattambi	9
PTB-25	-do-	Pattambi	9
PTB-28	-do-	Pattambi	9
PTB-31	-do-	Pattambi	9
PTB-1	-do-	Pattambi	5

DNA isolation and PCR amplification

Genomic DNA of 16 genotypes was isolated by modified IRRI protocol (21). The quality of the DNA was checked on an agarose gel (0.8%, w/v). Eighty SSR markers which are distributed on entire rice genome were used for this study. SSR primers were obtained from sigma Aldrich, Bangalore. The PCR reactions were performed in 10-µL volumes using eppendorf Master cycler Gradient. The reaction mixture contained 25 ng template DNA, each 0.5uM of forward and reverse primers, 125 uM dNTPs, 1x PCR buffer (20 Mm Tris HCl, 15mM MgCl2), and 0.05U/ul Taq DNA polymerase. The amplification profile was 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min with a final extension of 7 min. at 72°C. Amplified PCR products were electrophoretically resolved on a 3% agarose gel using 1x TBE buffer. DNA banding patterns were visualized using Syngene Bio-Imaging gel documentation system.

Data Analysis

Only clear and unambiguous SSR markers were scored. All the genotypes were scored for the presence (1) and absence (0) of the SSR bands. And the data was entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis. The excel file containing the binary data was imported into NT Edit of NTSYS- pc 2.02 (Rohlf 1999). Genetic similarities were estimated from the matrix of binary data using Jaccard coefficient. The resultant similarity matrix was employed to construct dendrogram using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic Means (UPGMA) to their genetic relationships and phylogeny.

Polymorphic information content

The term polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within a population, depending on the number of detectable alleles and the distribution of their frequency. In the present study, PIC value of a marker was calculated according to a simplified version after Anderson *et al* (3):

$$PICi = 1 - €^{n}j{=}1 \quad P^{2}ij$$

Where Pij is the frequency of the jth allele for the ith marker and summed over n alleles.

Results and Discussion

The results of phenotypic response of rice genotypes to brown plant hopper screening at the flowering stage (> 100 hoppers/hill) indicated the varied genotypic responses. The genotypes were classified into five groups from very slight drying (score 1) to all plant apparently dead (score 9), where first and second leaves of most plants partially yellowing (score 3), Pronounced yellowing and stunting or about half of the plants wilting or dead (score 5) and More than half of the plants wilting or dead and remaining plants severely stunted or drying (score 7). Among the 16 rice genotypes- PTB 33 widely used as donor parent for BPH by rice breeders and BM 71 scored as 1. Most of the cultivated varieties and land races were scored as 9 except PTB 1 and PTB18 which showed score 5 and 3 respectively.

Number of alleles and polymorphism information content values of SSR markers for 16 rice genotypes were showed in Table 2.The lowest amplicon size produced by RM 470 (83bp) while highest amplicon size belonged to RM 18606 (534bp). Out of eighty markers used, 37 SSR markers showed polymorphism by revealing 88 alleles. The number of alleles per locus varied from 2 (RM 17, RM 511, RM 470, RM 549, RM 341 etc) to 4 (RM 316) with an average of 2.37. Many studies have also reported significant differences in allelic diversity among various microsatillite loci (10,13).

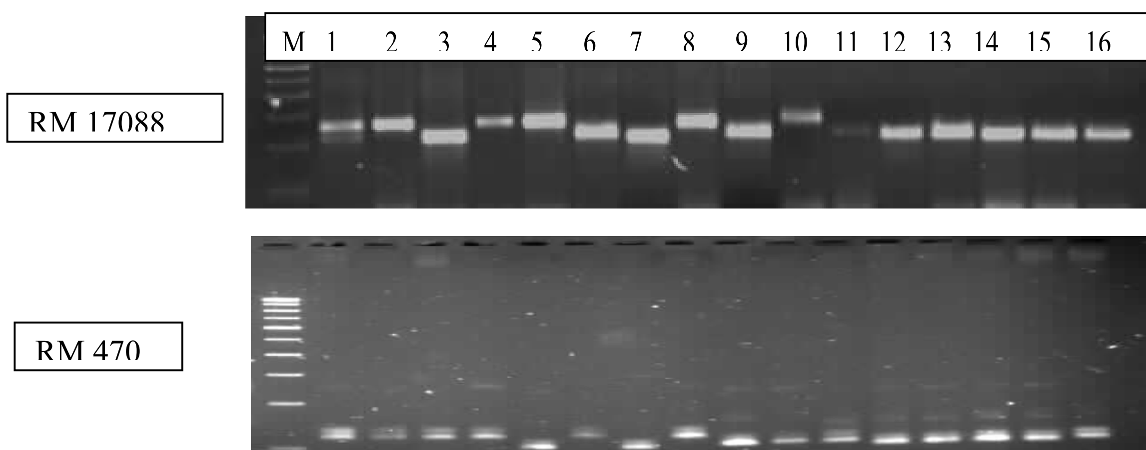
Table 2. Number of alleles and polymorphism information content (PIC) value of SSR markers for 16 genotypes

Marker	No.of alleles	PIC value	Chromosome No.	Amplicon size	Repeat motif
RM3	3	0.98	7	145	GA(25)
RM17	2	0.97	12	184	GA(21)
RM19	2	0.97	12	226	ATC(10)

RM84	3	0.98	1	113	TCT (10)
RM169	2	0.94	5	167	AG(12)
RM206	3	0.97	11	147	AG(33)
RM240	2	0.94	2	132	CT(21)
RM245	2	0.95	9	150	CT(14)
RM257	2	0.97	9	147	AG(30)
RM258	2	0.93	10	148	GA(21)
RM280	2	0.96	4	155	AG(11)
RM316	4	0.97	9	192	GT(8),TG(9) TTG(4),TG(4)
RM334	2	0.96	5	182	CTT(20)
RM336	3	0.97	7	154	CCT(18)
RM340	3	0.98	6	163	AAG(10)
RM341	2	0.97	2	172	CTT(20)
RM404	3	0.98	8	236	AG(29)
RM418	2	0.97	7	283	
RM428	2	0.96	1	266	AG(15)
RM470	2	0.96	4	83	AAG(14)
RM483	3	0.92	8	325	AT(26)
RM490	2	0.95	1	101	CT(13)
RM496	3	0.97	10	267	AG(24)
RM511	2	0.97	12	130	ACG(7)
RM520	2	0.98	3	247	AG(10)
RM527	2	0.96	6	233	AG(17)
RM541	2	0.97	6	158	TC(16)
RM549	2	0.98	6	148	CCG(9)
RM564	2	0.95	4	228	GT(14)
RM5864	2	0.96	3	134	ATC(8)
RM6100	2	0.97	10	173	ACG(8)
8278	2	0.97	1	144	AG (12)

10956	2	0.97	1	140	AAT(8)
11818	2	0.97	1	131	AG (14)
11968	2	0.96	1	441	AAT (22)
17088	3	0.98	4	251	AG(31)
18606	3	0.98	1	534	CCG(5)

The Polymorphic information content (PIC) value , a reflection of allele diversity and frequency among the cultivars , also varied from 0.92 to 0.98, with an average of 0.95. The polymorphic banding pattern of RM 470 and RM 17088 markers in 16 rice genotypes are presented in Figure 1.The genetic diversity of each SSR locus appeared to be associated with the number of alleles detected per locus. The higher the PIC value of the locus , higher the number of alleles detected. This observed pattern was consistent with report of Yu *et al*.(21) .



1.Sambamashuri	5.Dhanya Lakshmi	9.PTB 33	13.PTB 25
2.Prabhat	6.Deepthi	10.PTB 18	14.PTB 28
3.Swarna	7.MO1	11.PTB 21	15.PTB 31
4.improved samba	8.BM 71	12.PTB 22	16.PTB 1

Fig. 1: Polymorphism observed using RM 17088 and RM 470 in the 16 rice genotypes

Loci amplifying di-nucleotide repeat motifs were found to be more polymorphic with an average value of 2.45 alleles, than those with tri-nucleotide repeat motif which gave an average value of 2.26 alleles. Among the loci with perfect and compound di nucleotide repeat motifs, markers with a AG repeat motif showed the largest variability. These results suggest that the

total repeat count of SSR loci is associated with the number of alleles. The larger the repeats number in the microsatellite DNA, the larger the number of identified alleles. These results were consistent with those reported by Cho *et al*.(4) and Ni *et al*.(11) . However, in recent years, SSRs have become the marker of choice for genetic diversity analysis of breeding material .

Cluster analysis of the 16 rice genotypes based on SSR data divided the genotypes into three groups (Figure 2) with additional sub groups with in each group at a similarity coefficient level of 0.54. This dendrogram revealed that the genotypes derived from a genetically similar type clustered together. Group 1 comprised only of BM 71 which was scored as 1 is derivative of indica / japonica cross, whereas group 2 and 3 comprised the indica genotypes. Genotypes in Group 2 (PTB 33 and PTB 18) are of interest to rice breeders because they were scored as 1 and 3 respectively. Group 3 was again sub grouped into two. One group at a similarity coefficient of 65% consisted of 6 genotypes. Genotypes in this group were mostly land races and were susceptible to brown plant hoppers. Land races are known for resistance to major pest and diseases but in this study it was observed that there was considerable amount of resistance reaction to BPH among Pattambi rice genotypes (from score 1 to score 9). The parental phenotypic evaluation is in coincidence with genotypic cultivars using SSR markers. The other group consisted 7 genotypes of mostly cultivated varieties with varying level of similarity .Between the two rice subspecies, indica gave more alleles than japonica and likewise displayed a higher genetic diversity. These findings were consistent with those reported by Victoria C. Lapitan *et al*. (17).

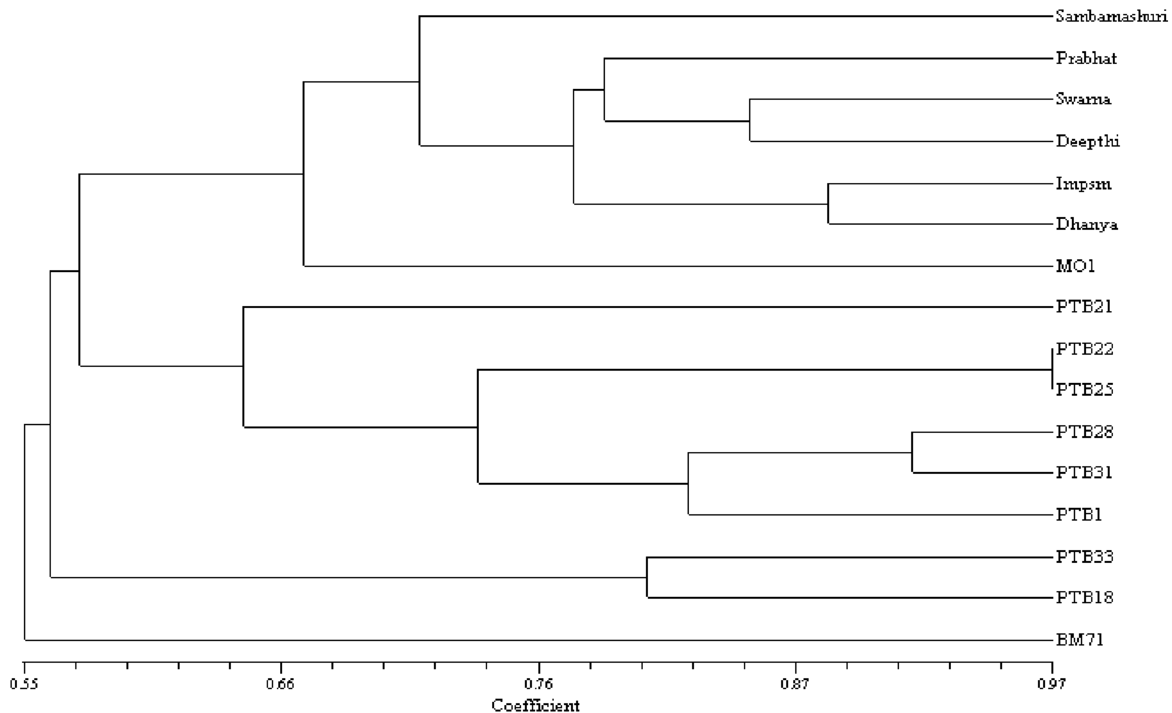


Fig.2. Dendrogram of 16 rice genotypes derived from UPGMA cluster analysis using jaccard coefficient based on 41 polymorphic SSR markers.

In summary, the accurate evaluation of genes in breeders' germplasm is of great importance for the selection of parental lines and development of new breeding populations. Having gene information for specific target loci (deduced from markers) can be extremely useful for breeders to efficiently use germplasm. The present study provided an overview of the genetic diversity of the 16 rice cultivars for brown plant hopper resistance.There was large range of similarity values for related cultivars using micro satellites provide greater confidence for the assessment of polymorphism. Since the SSR markers are neutral and co dominant, they are powerful tools to asses the genetic variability of the cultivars under study .The information about genetic diversity of these cultivars will be very useful for proper selection of parents and rice breeding programs especially for gene mapping and eventually for the application of marker assisted selection (MAS).

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Inhibition of Tumor Growth and Angiogenesis by an Aqueous Extract of *Terminalia bellirica*

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Abstract

The fruit of *Terminalia bellirica,* possess numerous medicinal properties and is used in Indian traditional system of medicine since ancient times. In the light of above ethno-medicinal values of *T.bellirica,* in this study we investigated the antiangiogenic activitiy of different solvent extracts of *Terminalia bellirica* fruit pericarp (TbFP) using Ehrlich ascites tumor (EAT) model, of which the TbFP aqueous extract (TbFP-Ae) showed highly promising results. In order to grow and metastasize, the tumor cells stimulate the development of new blood vessels through a process known as angiogenesis. Vascular endothelial growth factor (VEGF) promotes angiogenesis, which is found to be elevated in majority of cancers. Our results indicate that, TbFP-Ae inhibits in vivo, the growth of tumor cells in the peritoneal cavity of mice, as measured by body weight, ascites formation and tumor cell number. The peritoneum of mice treated with TbFP-Ae also showed significant reduction in peritoneal angiogenesis, which was further confirmed by inhibition of neovascularization in chorioallantoic membrane (CAM) assay. Quantitation of VEGF using the ascitic fluid from TbFP-Ae treated mice showed significant reduction in VEGF secretion when compared to untreated controls.. Additionally we noted the attenuaion microvessel density (MVD) count in histological section of mice peritoneum. This is the first report indicating the presence of an antiangiogenic biomolecule in *T.bellirica.*

Key words: *Terminalia bellirica*, ascites tumor growth, peritoneal angiogenesis, VEGF, microvessel density .

Introduction

Terminalia bellirica (Gaertn) Roxb is a large deciduous tree, the fruits of which possess numerous medicinal properties and is used as laxative, astringent, rejuvenative, cardioprotective, antacid, antioxidant and antibacterial (1). *Triphala,* a botanical and an ayurvedic preparation comprises of an equal amount of three herbal fruits, *Emblica offcinalis, Terminalia chebula* and *Terminalia bellirica* is referred as *'Mother of all healings'*. However the antitumor effect of *Terminalia bellirica* has not been paid much attention and needs to be investigated.

In order to grow and metastasize, the tumor cells should stimulate the development of new blood vessels through a process known as angiogenesis. Unlike normal blood vessels, tumor blood vessels are chaotic, irregular, and leaky, leading to an uneven delivery of nutrients and therapeutic agents to the tumor (2). The viability of tumor cells also dependent on the nutrients provided by the vasculature**.** Hence inhibitors of angiogenesis will starve tumor cells and block

tumor growth (3) making this process a major target for therapeutic intervention. The principal growth factor that controls angiogenesis is (VEGF). The secretion of VEGF is found to be elevated in a majority of cancers (4,5,6) and hence VEGF is used as prognostic indicator in tumor conditions (7,8,9,10). The expression of VEGF increases angiogenesis, which in turn increases microvessel density (MVD). MVD is used as a surrogate measure of angiogenesis in pathological specimen and tumor models (11). MVD in the peritoneal sections is *in vivo* indication of proliferation of endothelial cells and neovascularization.

An *in vitro* model system like chorioallantoic membrane of chick egg is used to validate compounds for their antiangiogenic activity in non-tumor context (17). An ascites tumor growing in peritoneal cavity of mice offers a good model for validation of antiangiogenic efficacy of novel biomolecules. Ehrlich Ascites Tumor (EAT) cells are spontaneous murine mammary adenocarcinoma cells, adapted to ascites form and carried in outbred mice by serial intraperitoneal (i.p) passage. Once EAT cells are injected i.p it takes about 15 days for the tumor to develop completely. During, this process the growing EAT cells secrete ascites fluid. Due to rapid growth of tumor cells and ascites burden, the animal succumbs to death within 15 days. It has been earlier reported that the vascular permeability factor (VPF) which is also known as (VEGF) is the key player in tumor angiogenesis and is secreted by (EAT) cells into the ascitic fluid (12). As a consequence, the inner lining of peritoneum shows extensive angiogenesis, which is the growth of new blood vessels.

Currently available chemotherapeutic anti-tumor drugs although effective in reducing cancer risks; lead to development of resistance in cancer Cells and patients often experience several adverse side effects (13, 14). In this context, natural compounds from plant kingdom plays a major role and form good replacement. In the light of above ethno-medicinal values of plants, in this paper we have used EAT model system in order to identify the antiangiogenic bio-molecule from *Terminalia bellirica* extracts. Our results indicate that the identified *Terminalia bellirica* fruit pericarp aqueous extract contained the antiangiogenic molecule and at molecular level the biomolecule inhibited proliferation of EAT cells, peritoneal angiogenesis, VEGF production and peritoneal microvessel MVD.

Materials and Methods

Terminalia bellirica fruits were collected in and around Mysore, India and identified by Botanist. The voucher specimen of the collected plant material was deposited and voucher number UOM.BOT.4820 was obtained from the Department of Botany, University of Mysore, Mysore. Swiss albino mice were obtained from Department of Zoology, University of Mysore, Mysore with the approval of institutional animal ethics committee and experiments were conducted according to guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, India. Fertilized hen's eggs were procured from the Government poultry farm, Bangalore. The chemicals and solvents were of analytical grade and purchased from Sisco Research Laboratory (SRL), Mumbai, India.

Polarity-based fractionation of TbFP

The pericarp of *Terminalia bellirica* fruits were separated, shade dried and powdered. Pulverised plant material was used for extraction

with different solvents of increasing polarity, *viz.,* hexane, benzene, chloroform, ethyl acetate, acetone, alcohol, methanol in soxhlet extractor till exhaustion and finally with distilled water on magnetic stirrer for 24h. The solvents were evaporated using rotatory evaporator under reduced pressure of 20-22 mmHg, lyophilised, and tested for antiangiogenic activity. Extracts (33.3mg), free from solvents were dissolved in 100µl of 0.1% DMSO from which 100µl was diluted (1:1) with saline and was subsequently used for the assays.

In vivo EAT cell growth and TbFP treatment

Nine groups of Swiss albino mice, each group containing 5 animals was included in the study. The animals were of six to eight weeks age, weighing about 25-30g. Ehrlich ascites tumor (EAT) cells, were maintained in our laboratory by i.p transplantation as described previously (15). In brief, 5 x 10^6 EAT cells/ mouse were injected i.p. EAT cells exhibits an exponential growth period from 6th or 7th day after tumor injection and the animal succumb to death on 12th to 14th day due to tumor burden. Each solvent extract 33.3mg was injected i.p into the tumor bearing mice, every alternate day after 5 days of tumor growth and the weight of the animals was monitored daily from the 1st day of transplantation till the 12th day of tumor growth. The mice were sacrificed on the 13th day and observed for peritoneal angiogenesis, secretion of ascites, cell number, microvessel density and secretion of VEGF.

Ascites volume, cell number and peritoneal angiogenesis

After sacrificing the untreated and the TbFP extracts treated EAT bearing mice, a small incision was made in the abdominal region and EAT cells along with ascites fluid were collected into a sterile polypropylene tube containing 2ml of saline and centrifuged at 3000rpm for 10min at 4ºC. Volume of ascites was calculated by subtracting the volume of the saline previously added from the supernatant. The cell number was determined by tryphan blue exclusion method using hemocytometer. After collection of cells along with the fluid, the incision on the abdomen wall was extended and exposed peritoneum was examined for vascularization and photographed.

Chorioallantoic membrane (CAM) assay

The chorioallantoic membrane assay is a well established assay and widely used to assess angiogenesis and antiangiogenesis (16). The fertilized eggs were incubated at 37ºC in a humid atmosphere for 10 days. A small window was made on the shells under aseptic condition to verify development of embryo. The window was resealed and the incubation was continued under the same conditions. On the 12th day, the window of the eggs were reopened and sterile cover slips containing air-dried saline or recombinant VEGF (50ng/egg) and TbFP solvent extracts were inverted over the CAM, resealed and returned to incubation for another 2 days. On the 14th day the windows were reopened and inspected for development of neovascularization in the area below the coverslip and photographed.

Quantification of VEGF

The quantification of VEGF was carried out by enzyme linked immunosorbent assay (ELISA) and VEGF was estimated in ascitic fluid collected from both untreated and TbFP extracts treated mice as described previously (17). In brief, 100µl of ascitic fluid from TbFP solvent extracts treated and untreated EAT bearing mice were coated onto 96 well microplates using coating buffer (50mM Na_2CO_3, pH. 9.6) and incubated overnight at 4^0C, wells were washed and blocked using skimmed milk followed by incubation with anti-VEGF

antibodies. The wells were washed and probed with secondary antibody tagged to alkaline phosphatase. *P*-NPP was used as substrate and absorbance was measured at 405nm with medispec ELISA reader.

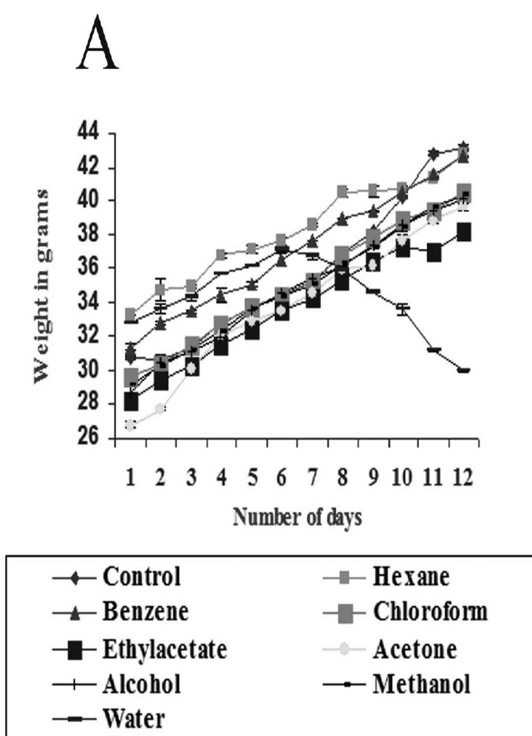
H&E staining and Microvessel density (MVD)

The peritoneum of the mice treated with or without TbFP extracts was fixed in formalin, dehydrated with alcohol and embedded in paraffin. The 5μm sections were taken using microtome and stained with routine hematoxylin and eosin stain. MVD was determined by 'hotspot' method (11) using Nikon binocular microscope. In brief 10 fields with highly vascularized areas were screened at low magnification (10x), and further magnification was changed to high-power field (HPF) (40x) and the microvessels were counted.

Results

In vivo Effect of TbFP extracts on EAT cell growth, ascites secretion and Cell number

The effect of different solvent extracts of TbFP on EAT cell number, ascites volume is provided in Tabel-1. EAT cells (5 x 10^6 cells) injected / mouse on the day of transplantation increased to an average of 1.83 x 10^8 cells/mouse at the end of the growth period in untreated animals. Animals, which received (TbFP-Ae) showed 2.8 folds reduction in the EAT cell number (0.65 x 10^8 cells/ mouse) compared with that of untreated animals. Whereas no significant reduction in EAT cell number was observed in other solvent extracts treated mice (Fig-1C). This reduction in cell number by TbFP-Ae reflected on the body weight of the animals. From Fig-1A, it is evident that the untreated and solvent extracts treated mice showed continuous increase in the body weight from the day of transplantation till 12th day. The mice that received TbFP-Ae showed an average 80.78% reduction of body weight from the 5th day. EAT cells grow as ascites tumor by accumulating large amount of ascites fluid (8.55ml), when injected intraperitoneally to mice. The *in vivo* effect of TbFP solvent extracts on secretion of ascites in EAT bearing mice is shown in table-1 and depicted in Fig-1B. The volume of ascites formed due to tumor induction decreased upon TbFP-Ae treatment to an extent of 75.43% (2.0±0.10ml) when compared to that of untreated EAT bearing animals (8.5±0.15ml). Other solvent extracts did not have any effect on either growth of EAT cells or formation of ascites.



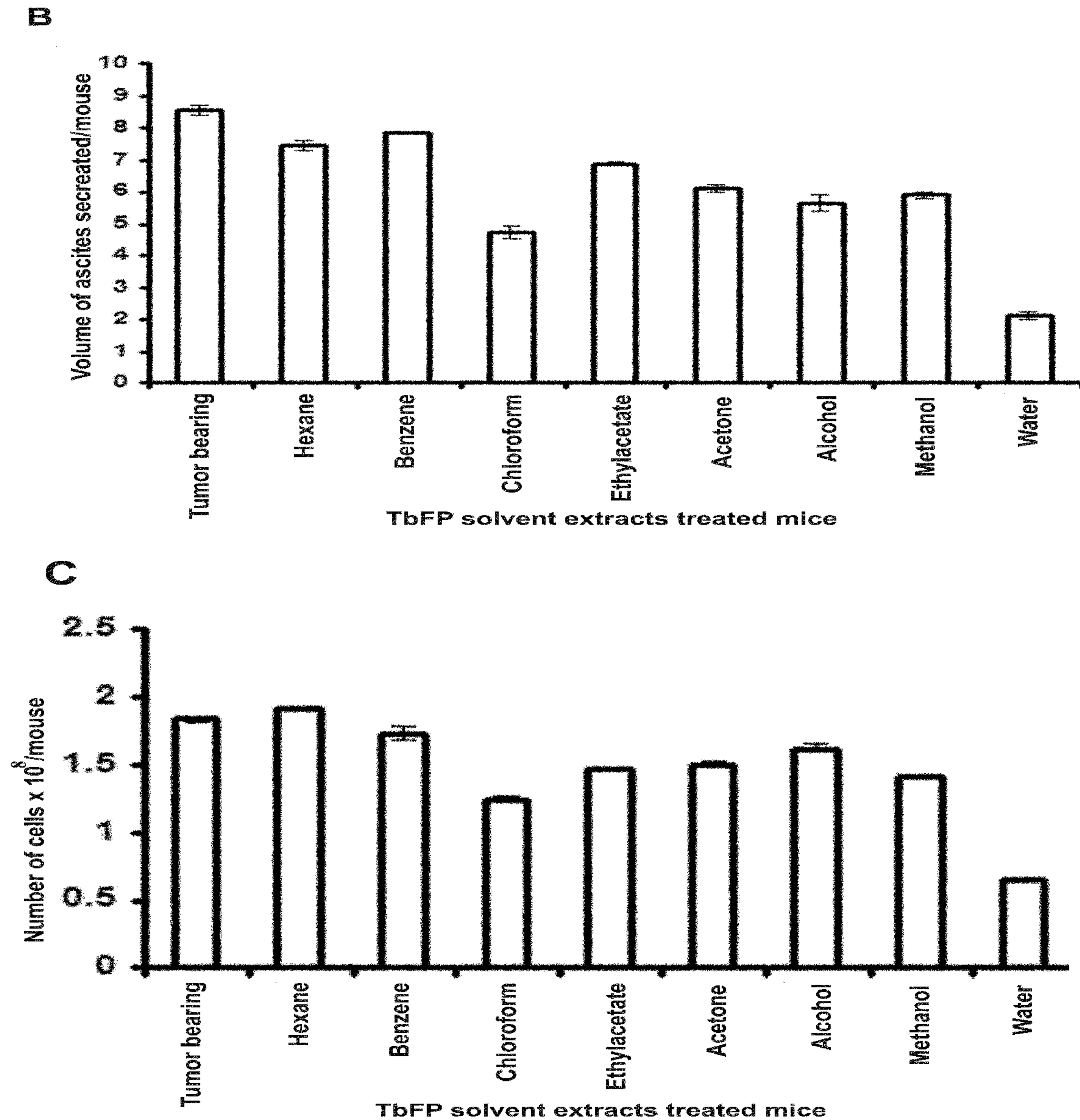


Fig. 1: Effect of different solvents extracts of *T. bellirica* on EAT cell growth, ascites volume and cell number *in vivo*. EAT cells (5x 10^6) were injected i.p into mice and from 6th day of transplantation the mice were treated with or without solvent extracts of *T. bellirica* and body weight of both control and treated groups of the animals were monitored daily and graph was plotted. B. The volume of ascites secreted by mice treated with or without solvent extract. C. The cell number was determined by tryphan blue exclusion method. The above results are the average of 3 experiments and means of 5-animals/ group.

Table-1: Average EAT cell number counted using hemocytometer, ascites volume, number of microvessel density count/high power field of peritoneal section stained with haematoxylin and eosin stain and VEGF secreted in different solvent extracts treated EAT mice *in vivo*. (Avg- average, MVD-Microvessel density, ng ml^{-1}- nanogram/millilitre, VEGF-Vascular endothelial growth factor.

Solvent extract treated	Avg. EAT cell number/ mouse	Avg. Ascites volume secreted/mouse (ml)	Avg. MVD/ HPF	VEGF (ng ml^{1})
Untreated	1.83	8.55	18.09	1200.9
Hexane	1.9	7.45	15.31	1831.31
Benzene	1.73	7.85	18.32	1219.13
Chloroform	1.24	4.70	9.62	738.33
Ethylacetate	1.48	6.85	17.11	1190.01
Acetone	1.50	6.10	13.44	899.08
Ethanol	1.62	5.65	12.03	700.31
Methanol	1.41	5.91	10.19	363.09
Water	0.65	2.10	3.21	21.34

Angio-inhibitory effect of TbFP extracts

The peritoneum of untreated EAT bearing mice showed extensive angiogenesis, while TbFP-Ae treated mice showed considerable reduction in the peritoneal angiogenesis. Those mice, which received other solvent extracts, did not show any significance in reducing peritoneal angiogenesis (Fig-2A). Further, CAM assay proved that TbFP-Ae inhibited the formation of new blood vessels. The results of the CAM assay showing the inhibition of angiogenesis in comparison with that of untreated and other solvent extracts treated CAM are provided in Fig-2B.

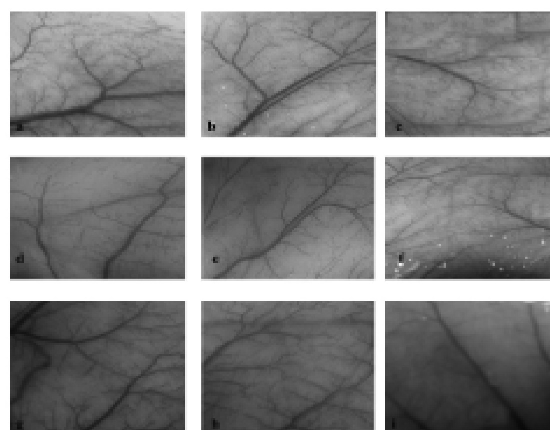


Fig 2A

Fig. 2A: Representative photographs of mice peritoneum a. untreated, b. Hexane, c. Benzene, d. Chloroform, e. Ethylacetate, f. acetone, g. ethanol, h. methanol and i. water extract. After 12th day the untreated and TbFP different solvent extracts mice were sacrificed and the peritoneum was observed for neovascularization. From the figure it is evident that the formation of blood vessels in the peritoneum of aqueous extract treated mice was extensively inhibited compared to the vascularization in peritoneum of untreated and other extracts treated mice.

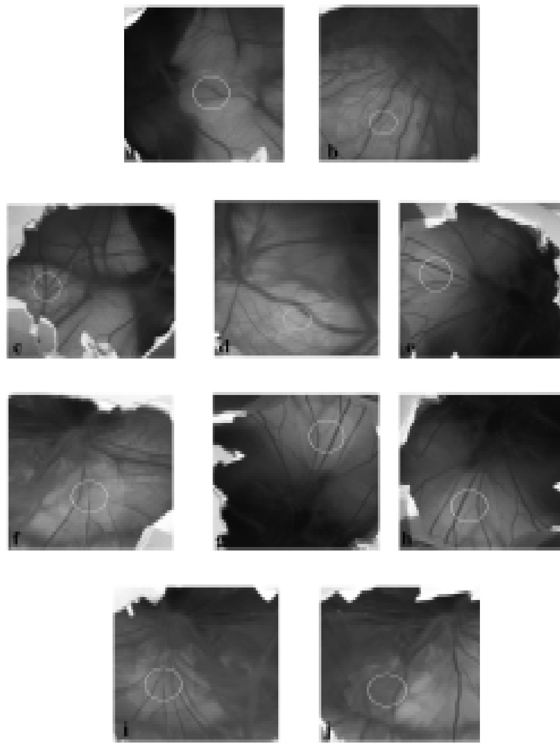


Fig 2B

Fig. 2B: Chorioallantoic membrane (CAM) assay. a. Untreated, b. VEGF, c. Hexane, d. Benzene, e. Chloroform, f. Ethylacetate, g. acetone, h. ethanol, i. methanol and j. water extract, when blood vessels were observed under the cover slip, it was clear that the formation of new blood vessels were inhibited in the CAM treated with TbFP-Ae, compared to the untreated, VEGF and other solvent extracts treated CAM.

Effect of TbFP on production of VEGF

Quantification of VEGF over the tumor growth period of 12days showed 1200.9ng/ml of VEGF secreted by EAT cell bearing untreated mice. However, in the TbFP-Ae treated animals the estimated VEGF was 21.34ng/ml. These results clearly indicate that there was a reduction of 98.34% in the secretion of VEGF levels in the ascitic fluid of mice treated with TbFP-Ae when compared to untreated animals. There was no In contrast, there was no reduction in VEGF levels in mice treated with other solvent extracts (Table–1).

Histological analysis and Microvessel density

The decreased secretion of VEGF in TbFP-Ae treated mice in turn reflected on the reduction in formation of blood vessels. The microvessel density was counted in the peritoneum section of TbFP extracts treated and untreated tumor-bearing mice. In untreated mice the average MVD/HPF was 18.09±0.02 and in TbFP-Ae treated mice it was 3.21±0.13. This accounted for the reduction of MVD by 82.2% in TbFP-Ae treated mice peritoneum. The representative photomicrograph of peritoneal sections of untreated and TbFP extracts treated mice are shown in Fig.3 and is further emphasised in Table 1.

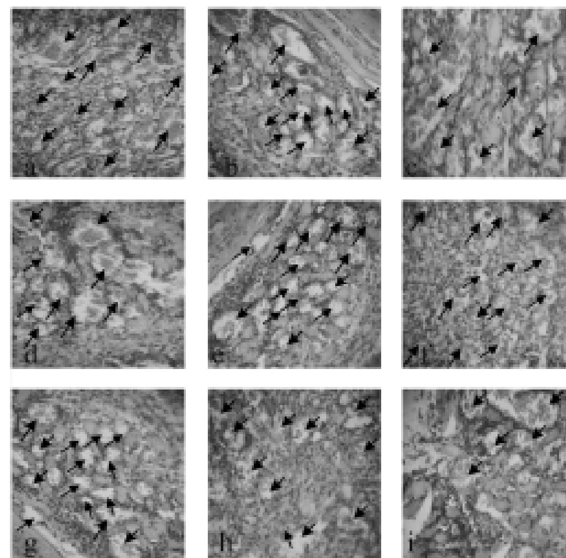


Fig. 3: Representative photomicrographs of 5µ H&E stained mice peritoneal. Sections of control and treated with different solvent extracts of *Terminalia bellirica*. a. Untreated, b. hexane, c. benzene, d. chloroform, e. ethylacetate, f, acetone, g. alcohol, i. water extracts treated mice. Reduction of microvessels in water extract is evident from the above figure.

Discussion

Angiogenesis is a major pathological component of a grave disease such as cancer. Antiangiogenic drugs have been shown to decrease certain tumors in animal models and induce long-term tumor dormancy. Several successful attempts have been done to explore the antiangiogenic activity from plants. By using CAM assay for validation, Wang et al. (18) reports the antiangiogenic effect of aqueous extract from twenty-four herbs. *Terminallia bellirica,* selected in this investigation is one of the plants having ethno-medicinal value and has been used in Indian traditional medicine. Polarity-based fractionation of TbFP proved that, the aqueous extract possessed highly promising antiangiogenic property in Ehrlich ascites tumor model. The EAT cell proliferation, ascites volume, tumor cell number, peritoneal angiogenesis, VEGF levels and microvessel density are biological events which offer measurable parameters to validate novel biomolecules with anti-tumor and antiangiogenic activities. This is the first report on *Terminalia bellirica* as an antiangiogenic component in mouse mammary carcinoma model. Methanol extract of stem bark *Bombax ceiba* has been shown to inhibit the tube formation in HUVEC cells (19). This being an *in vitro* assay it does not reflect on the *in vivo* antiangiogenic activity of *Bombax ceiba*. Similar to the data presented in this paper on inhibition on the development of new blood vessels in CAM assay by TbFP-Ae. Jung et al. (20) have found that the methanol extract of *Ulmus davidiana* displayed a strong inhibition of neovascularization in chick membrane. A decreased microvessel density in peritoneum of EAT bearing mice by curcumin confirmed its antiangiogenic property from studies made by Belakavadi et al., (17). The data on TbFP-Ae inhibition of tumor induced peritoneal angiogenesis clearly indicates that the extract contains a potent antiangiogenic biomolecule. In the present investigation the inhibition of EAT cell growth, *in vivo* by TbFP-Ae supports to the earlier findings that the aqueous extracts of *Acanthus ilicifolius*, *Alternanthera tenella* and *Glycyrrhiza glabra* plants inhibits growth of EAT cell (21,22,23). At molecular level, the mechanism of antiangiogenesis by TbFP-Ae involves, inhibition of the secretion of VEGF by 98.34%. Periyanayagum et al. (24) also reports that, the aqueous extract of *Justicia gendarussa* leaves inhibits angiogenesis in chorioallantoic membrane at concentrations of 25 μg, 50 μg and 100 μg. A dose of 0.1% (w/w) aqueous extracts of *Rubus suavissimus* caused 41% inhibition of angiogenesis when compared with saline treated human- tissue based fibrin-thrombin clot assay (25). The present study gains more importance as it includes both quantitative and qualitative validation of angiogenesis. Findings from this study indicate the presence of an antiangiogenic biomolecule in *Terminalia bellirica* fruit pericarp aqueous extract. This observation warrants further study to isolate and characterized the bioactive compound from this plant. As it is easily available cost effective, medicinal plant, it might form a new arsenal in antiangiogenic dependent therapy.

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Microwave Synthesis of some new Quinazolinone Formazans for their Antimicrobial and Antihelminthic Activities

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Abstract

Halogenated hetrocyclic compounds have a wide range of pharmacological activities such as antimicrobial, analgesic, anti-inflammatory and hypoglycemic activities. In the present study 1-halo substituted phenyl-3-halo substituted phenyl-4-[benzamido-(2-methyl-3-quinazoline)-4-one] formazans were synthesized by microwave irradiation and conventional heating. Synthesized compounds were screened for antimicrobial and antihelminthic activities. The title compounds were characterized by FTIR, ^{1}HNMR and mass spectroscopic analysis. Selected compounds possess significant antimicrobial and antihelminthic activities. The compounds were synthesized by microwave irradiation in 1.5 to 6 minutes.

Key words: Quinazolinone formazans, Microwave irradiation, Antimicrobial, Antihelminthic

Introduction

Of the large number of heterocyclic systems known today the nitrogen heterocycles are of great importance as they are present in nucleic acids, vitamins, proteins and other biological systems. Among the wide variety of nitrogen heterocycles that have been explored for developing pharmaceutically important molecules, the compounds bearing quinazoline nucleus found to possess antibacterial, antifungal(1, -5), analgesic (6) and anti-inflammatory activities (7). In recent years the applications of microwave irradiation in organic synthesis are increasing very rapidly due to advantages like short reaction time, suppression of side products, less pollution processes and at the same time excellent yields (8, 9). In view of remarkable biological activity of quinazolines we have synthesized new quinazolinone formazans for their antimicrobial and antihelminthic activity.

Materials and Methods

Step-1: Synthesis of 2-methyl – benzoxazine – 4-one:

A mixture of 2-amino benzoic acid (anthranilic acid, 0.1 mole) and acetic anhydride (25 ml) were refluxed for 2hrs by conventional heating or for 5 min by microwave irradiation respectively. The reaction mixture was poured into crushed ice. The resulting mass was filtered and washed for several times.

Step- 2: Synthesis of 3-(4-carboxy phenyl)-2- methyl – 3 – quinazoline -4-one:

To a solution of 2-methyl – benzoxazine – 4-one (0.01 mole) in alcohol, para amino benzoic acid (PABA) was added and refluxed for 4 hrs by conventional heating or for 5 min by microwave irradiation respectively. The resulting product was added to crushed ice and filtered.

Step- 3: Synthesis of 3-(4- benzoyl chloro)-2-methyl – 3-quinazoline - 4-one:

To the solution of 3-(4-carboxy phenyl)-2- methyl – 3 – quinazoline -4-one in alcohol (0.01 mole) double the molar concentration thionyl chloride (0.02 mole) was added. The resulting mixture was concentrated and the solid residue formed at the bottom was collected.

Step- 4: Synthesis of 3-(4- hydrazino benzoyl)-2- methyl -3-quinazoline -4-one:

To the solution of 3-(4- benzoyl chloro)-2-methyl – 3-quinazoline - 4-one (0.01 mole) in alcohol double the molar concentration (0.02 mole) of hydrazine hydride is added, refluxed for 4 hrs by conventional heating and 4 minutes by microwave heating. The resulting product was concentrated and the residue was collected and dried.

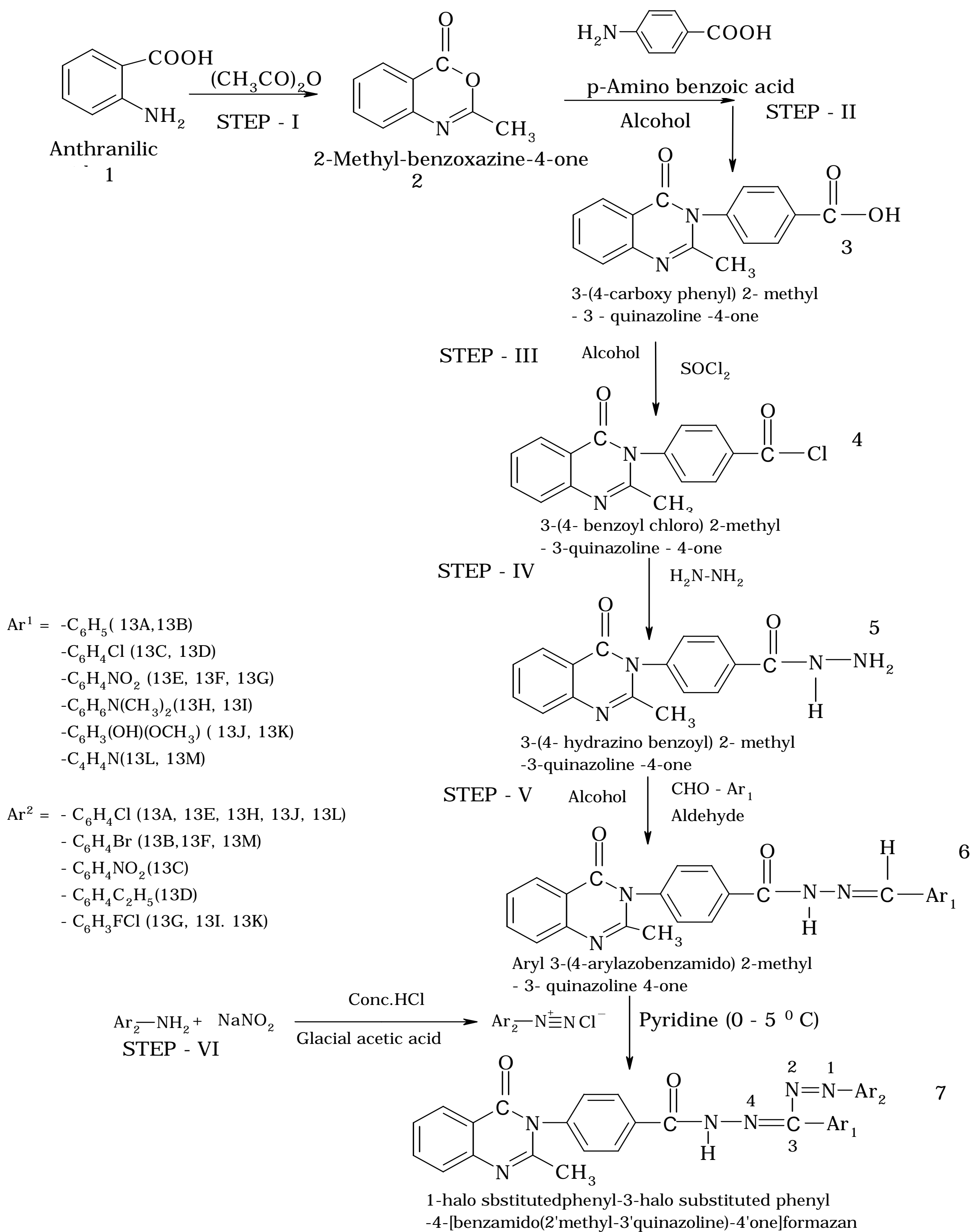
COOH
NH2
Anthranilic
1
(CH3CO)2O
STEP - I
2-Methyl-benzoxazine-4-one
2
H2N COOH
p-Amino benzoic acid
Alcohol
STEP - II
3-(4-carboxy phenyl) 2- methyl
- 3 - quinazoline -4-one
3
STEP - III
Alcohol
SOCl2
4
3-(4- benzoyl chloro) 2-methyl
- 3-quinazoline - 4-one
STEP - IV
H2N-NH2
5
3-(4- hydrazino benzoyl) 2- methyl
-3-quinazoline -4-one
STEP - V
Alcohol
CHO - Ar1
Aldehyde
6
Aryl 3-(4-arylazobenzamido) 2-methyl
- 3- quinazoline 4-one
Ar1 = -C6H5(13A,13B)
-C6H4Cl (13C, 13D)
-C6H4NO2 (13E, 13F, 13G)
-C6H6N(CH3)2(13H, 13I)
-C6H3(OH)(OCH3) (13J, 13K)
-C4H4N(13L, 13M)
Ar2 = - C6H4Cl (13A, 13E, 13H, 13J, 13L)
- C6H4Br (13B,13F, 13M)
- C6H4NO2(13C)
- C6H4C2H5(13D)
- C6H3FCl (13G, 13I. 13K)
Ar2—NH2 + NaNO2
STEP - VI
Conc.HCl
Glacial acetic acid
Ar2—N≡N Cl⁻
Pyridine (0 - 5 0 C)
7
1-halo sbstitutedphenyl-3-halo substituted phenyl
-4-[benzamido(2'methyl-3'quinazoline)-4'one]formazan

Table No.1 Antibacterial and antifungal activity of 1-halo Substitutedphenyl-3-halo substituted phenyl-4-[benzamido(2'methyl-3'quinazoline)-4'one]formazans

Comp.	Antibacterial activity (zone of inhibition in mm)												Anti fungal activity (zone of inhibition in mm)							
	B. subtilis		*B. cereus*		*E. coli*		*S. aureus*		*S. epidermidis*		*Ps. aeruginosa*		*C. albicans*		*C. glabrata*		*A. niger*		*S. cerevecae*	
	5	**10**	**5**	**10**	**5**	**10**	**5**	**10**	**5**	**10**	**5**	**10**	**5**	**10**	**5**	**10**	**5**	**10**	**5**	**10**
13A	-	6	-	-	-	-	-	7	-	-	-	-	-	-	-	9	-	-	-	-
13B	-	7	-	6	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	-
13C	-	7	-	-	-	9	-	6	-	-	-	-	-	-	-	10	-	-	-	-
13D	-	8	-	11	-	12	-	10	-	-	-	9	-	-	-	6	-	-	-	-
13E	7	13	9	15	8	14	6	14	11	18	8	12	-	-	-	-	-	-	-	-
13F	11	19	8	13	-	11	10	19	11	19	-	10	-	6	8	19	-	9	-	6
13G	11	17	9	17	9	13	10	15	15	24	9	16	-	10	12	27	9	17	-	10
13H	13	25	10	17	11	18	-	10	13	21	13	25	8	14	9	15	10	12	10	13
13I	11	19	-	11	12	19	-	9	7	15	8	13	5	14	11	29	8	19	9	18
13J	-	7	8	11	12	16	9	12		-	7	9	-	9	8	14	11	15	14	15
13K	12	19	-	10	13	21	-	11	9	15	-	9	6	12	12	29	10	27	-	5
13L	-	8	-	7	-	12	8	13	-	-	9	10	-	-	8	19	-	7	-	-
13M	-	-	-	-	-	-	-	11	-	-	-	-	-	10	12	26	7	12	-	8
Ciprofloxacin	20		17		22		19		20		21		-		-		-		-	
Fluconazole	-		-		-		-		-		-		19		22		17		18	
Control (DMF)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(-) indicates no zone of inhibition

Step- 5: Synthesis of Aryl 3-(4-arylazobenzamido)-2-methyl – 3- quinazoline-4-one derivatives:

To a solution of 3-(4- hydrazino benzoyl)- 2-methyl -3-quinazoline -4-one (0.01 mole) in alcohol, aldehyde or substituted aldehyde (0.01 mole) was added, stirred well to get the respective quinazoline derivatives. The resulting product was filtered and dried.

Step- 6: Synthesis of 1-halo substitutedphenyl-3-halo substituted phenyl-4-[benzamido(2'methyl-3'quinazoline)-4'one]formazans:

The diazonium salts derived from the respective amines (0.01 mole) were added with stirring to Aryl 3-(4-arylazobenzamido)-2-methyl – 3-quinazoline-4-one derivatives in pyridine at 0-5ºc for 30 minutes. The reaction mixture was added to cold water was afforded title compounds (13A – 13M).

Antimicrobial activity

The synthesized quinazolinone formazans were screened for antibacterial activity against six bacterial strains: *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and antifungal activity against four fungal strains: *Candida albicans, Candida glabrata, Aspergillus niger, Sacromyces cerveceae* by paper disc diffusion method(10). The compounds were tested at two different concentrations (5mg/ml and 10mg/ml) against both the organisms. Ciprofloxacin, Fluconazole were used as standards for comparision. The zone of inhibition was measured and the results are depicted in Table 1.

Antihelminthic activity

The earthworms (*Pheritima posthuma)* were used to evaluate Antihelminthic activity(11). Albendazole and piperazine citrate were used as standards. The worms which becomes motionless was noted as paralysis time and the time taken for complete death of worms were also recorded and reported in table.2.

Table No 2: Antihelminthic activity of 1-halo substitutedphenyl-3-halo substituted phenyl-4-[benzamido(2'methyl-3'quinazoline)-4'one]formazans

S.No	Compound code	Paralytic (sec)	Lethal (sec)
1	13A	65±1.14	120±1.48
2	13B	65±1.10	110±0.95
3	13C	92±2.08	153±1.79
4	13D	85±2.02	138±1.52
5	13E	70±1.34	115±1.84
6	13F	65±0.49	128±1.30
7	13G	32±0.84	68±1.05
8	13H	48±1.48	71±1.26
9	13I	34±0.84	61±0.89
10	13J	47±1.26	64±1.30
11	13K	33±1.58	76±1.05
12	13L	60±2.08	136±1.38
13	13M	68±2.08	100±2.02

	Albendazole (10mg/ml)	21±0.89	53 ± 1.34
	Piperazine citrate (10mg/ml)	18±0.60	48±0.49
	Control (1%DMF in Normal Saline)	-	-

Physical Constant: Melting points were determined by open capillary method and the results are uncorrected. The reactions of the compounds were closely monitored by TLC and purified by column chromatography. The elemental analysis was carried out by Chem. Office software and the data obtained from Carlo Erba 1108 elemental analyzer.

Spectral analysis

The IR, ^{1}H FT-NMR (BRUCKER AMX 400 MHz) and mass values of title compounds are shown below:

1-(4-Chlorophenyl–3(4-nitrophenyl) – 4-[benzamido(2 – methyl – 3- quinazoline) – 4- one] formazan (13C)

IR 842.90 (1,4-disubstituted benzene), 1380.64 (aromaticC=C), 2842.38 (C-H, Hetero aromatic ring), 1521.74 (C=O, Ketone), 1596.10 (C=N, azomethine), 3432.83 (N-H), 1213.89(C-N aromatic),1106.69 (C-N aliphatic), 2929.80 (N=N) , 1343.68 (Ar-NO2), 682.25(C-Cl) Mass 560.5 (M^++1) ^{1}H NMR 7.261-8.716 δ (9H Ar-H), 3.495 δ (1H, NH)1.547 δ (3H, -CH3).

1-(3-Fluoro-4-chlorophenyl–3(4-nitrophenyl) – 4-[benzamido(2 – methyl – 3- quinazoline) – 4- one] formazan(13G):

IR 1013.34 (1,3,4-trisubstituted benzene), 844.11 (1,4-disubstituted benzene), 1479.02 (aromaticC=C), 1527.12 (C=O, Ketone), 1646.24 (C=N, azomethine), 3439.84 (N-H), 1210.11 (C-N aromatic),1045.55 (C-N aliphatic), 1344.53 (Ar-NO2), 678.86 (C-Cl), 947.74 (C-F), Mass 580.2

^{1}H NMR 7.261-8.715 ä (9H, Ar-H), 3.495 δ (1H, NH)1.549 δ (3H, -CH3).

1-(4-Chlorophenyl)-3-(4-dimethylaminophenyl)-4-[benzamido(2-methyl-3-quinazoline)-4-one]formazan (13H):

IR 810.77 (1,4-disubstituted benzene), 1433.17 (aromatic C=C), 1519.32 (C=O, Ketone), 1600.34 (C=N, azomethine), 2803.71 (C-H, Hetero aromatic ring), 2909.15 (N=N), 1362.38(-tertiary amine) , 1227.17 (C-N aromatic),1061.78 (C-N aliphatic), 604.39 (C-Cl),Mass 560.4,^{1}H NMR 6.707- 7.709 δ (9H, Ar-H), 3.033 δ (6H, N(CH3)2), 2.168δ (3H, -CH3).

1-(3-Fluoro-4-chlorophenyl–3(4-dimethyl-aminophenyl)–4-[benzamido (2 – methyl – 3-quinazoline) – 4- one] formazan(13I)

IR 1061.45 (1,3,4-trisubstituted benzene), 810.96 (1,4-disubstituted benzene), 1430.61 (aromaticC=C), 1518.91 (C=O, Ketone), 1600.34 (C=N, azomethine), 2370.58 (C-H, Hetero aromatic ring), 2909.60 (N=N), 1362.08(-tertiary amine) 3431.74 (N-H), 1227.33 (C-N aromatic),1045.55 (C-N aliphatic), 604.28 (C-Cl), 949.98 (C-F), Mass 579.4, ^{1}H NMR 6.701-7.707 δ (9H, Ar-H), 3.023 δ (6H, N(CH3)2).

1-(3-Fluoro-4-chlorophenyl)-3-(2-hydroxy-4-methoxy phenyl)-4-[benzamido(2-methyl-3-quinazoline)-4-one] formazan(13K):

IR 815.44 (1,4-disubstituted benzene), 1028.16 (1,2,4-trisubstituted benzene), 1508.09 (C=O, Ketone),1426.23 (aromatic C=C), 1599.03 (C=N, azomethine), 2374.25 (C-H, Hetero aromatic ring), 2927.78 (N=N), 3431.74 (N-H), 1279.94 (C-N aromatic),1028.16 (C-N aliphatic), 699.37 (C-Cl), 962.08 (C-F), Mass: 587.3, ^{1}H NMR 9.712 δ (1H, -OH), 6.627-7.471 δ(9H, Ar-H), 3.845 δ (6H, -OCH3) 2.513 δ (3H, -CH3).

Discussion

In present research work 13 novel quinazolinone formazans were synthesized by microwave irradiation and conventional methods. Microwave irradiation technique was obtained good yields in short period (5 min) in comparison to conventional heating. The structures of the compounds were characterized by FTIR, ^{1}HNMR, mass and elemental analysis. All synthesized compounds were active against all tested microorganisms when compared to standard drugs. Among the test compounds 13F, 13G, 13H, 13J and 13K were exhibited significant antimicrobial activity and compounds 13G, 13H, 13I and 13K possess better antihelminthic activity. The other compounds shown moderate activity.

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Antibacterial Properties of Secoisolariciresinol Diglucoside Isolated from Indian Flaxseed Cultivars

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Abstract

Secoisolariciresinol diglucoside (SDG) is an important lignan found in flaxseed and is an emerging source in the functional food area. In the present study, antibacterial properties of SDG extracts from hull, endosperm and flour fractions of Indian flaxseed (*Linum usitatissimum*) varieties (LVF-01 and GVF-03) were evaluated. The SDG extracts were tested against the six bacterial species *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Agrobacterium tumefaciens, Bacillus cereus,* and *Escherichia coli*. The maximum SDG and phenolic contents were found to be 16.9 and 12.5 mg/g and 3.18 and 2.70 mg/g in hull fractions of LVF-01 and GVF-03 respectively, when compared to flour and endosperm fractions. Among the fractions, the hull fraction of LVF-01 showed maximum activity 31.5 mm at MIC 100 ppm against *E. coli*, while minimum inhibitory activity was 3.1 mm with MIC at 300 ppm against *B. subtilis*. Similarly, in the case of GVF-03, maximum activity (31.9 with MIC 150 ppm) of the hull fraction, whereas, its minimum inhibitory activity was (2.3 mm, with MIC 350 ppm) against *B. subtilis*, when compared to endosperm and flour ractions.

Keywords: Flaxseed, lignans, SDG extracts, phenolics, HPLC, antibacterial properties

Introduction

Flaxseed (*Linum usitatissimum*) is the most valuable oil seed crop grown in several areas around the world. It is processed for its oil and meal. In flaxseed, the hull or seed coat is tightly adhered to the embryo and it is very difficult to separate unlike many other oilseeds in their pure form without oil extraction. The hull portion is rich in fibre and lignans, whereas, the endosperm is higher in oil and protein content. Flaxseed has gained importance in food industries as a component in designer food, functional food and in value added products because of its high content of lignans, which exert nutraceutical and therapeutic principles (1). Flaxseed is the richest source of phytoestrogen or plant lignan SDG

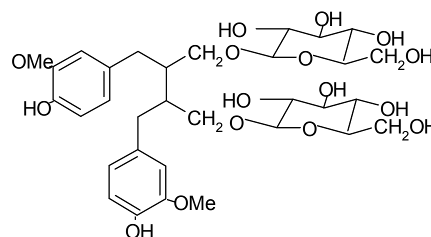

Fig. 1: Structure of SDG (2, 3-bis [(4-hydroxy-3-methoxyphenyl) methyl] -1, 4 butane-diglucoside) (Rajesha et al. 2008)

and constitutes about 75-800 times higher than vegetarian food sources (2). In addition to high content of SDG; mammalian lignan precursor, flaxseed is also well known for other lignan precursors such as matairesinol relatively in lower level (3, 4). Lignans are an important phytoestrogen with weak estrogenic and anti-estrogenic properties, and possesses diverse bioactivities. Epidemiological studies have reported the chemo preventive effects of lignans on tumors of colon, skin and mammary glands (5). SDG exhibits a wide range of health promoting activities, which is effective against the on set of various sort of cancers such as breast, colon and prostate (6, 7). The consumption of flaxseed based diet by rats caused protective effects against cardiovascular diseases such as reduction in the level of LDL cholesterol and aortic atherosclerosis (8). Flaxseed is well known for its hydroxyl radical scavenging activity of SDG and antioxidant activities (9) in addition to ED and EL *in vitro* (10). Lignans also exert antibacterial and cytotoxic activities, antitumor and antivirus etc., (11). The production of mammalian lignans ED and EL after flaxseed ingestion have been shown to inhibit aromatase activity and stimulate production of sex hormone binding globulin (SHBG), which is hypothesized to the reduction of endogenous estrogen level and lengthening of the estrous cycle in *in vitro* and animal models (12, 13).

There is growing awareness of flaxseed as a source of food and for several therapeutic purposes. Further, there is lack of information on antibacterial properties of SDG isolated from different fractions obtained upon its dehulling or milling process. Hence, the present study was under taken to evaluate the antibacterial properties of SDG isolated from hull, endosperm and flour against important some pathogenic bacteria.

Materials and Methods

Chemicals

All the solvents and chemicals used for the experiment were of analytical grade obtained by Sigma Chemicals Co., St. Louis , MO. USA. Solvents used for HPLC were of HPLC grade and purchased from Ranbaxy fine chemicals Ltd. Mumbai, India

Flaxseed

Two flaxseed cultivars, grown at two locations, Ranebennur and Gadag, North Karnataka, India were purchased from the local market. The University of Agricultural Sciences, Hebbal, Bangalore, Karnataka, India authenticated the seeds. The specimen samples of seeds LVF-01 and GVF-03 were preserved for analysis. Flaxseeds were processed by the combination of conditioning, de-hulling, sieving and aspiration. The dehulling of the seed was carried out using Kisan Krishi Yantra Udyog, Kanpur, India situated at Department of Grain Science and Technology, CFTRI, Mysore, India. The fractions such as hull, endosperm and flour were obtained after dehulling process.

Extraction of SDG from flaxseed

The extracts of SDG were prepared by the Klosterman method described by Rickard *et al.,* (1) from flaxseed fractions such as hull, endosperm and flour obtained upon dehulling process.

High performance liquid chromatography (HPLC) analysis of SDG in flaxseed fractions

High performance liquid chromatographic analyses were carried out and the SDG peaks were identified and quantified by comparison with those of the SDG standards, and its amount were also calculated as reported in our recent study (14).

Bacterial strains and culture conditions

The antibacterial activity was tested against *Staphylococcus aureus (*FRI 722*), Bacillus cereus* (F 4433), *Escherichia coli* (D 21) were obtained as generous gift from Dr. E. Notermans, National Institute of Public Health, Netherlands, Dr. J. M. Kramer, Central Public Health Laboratory, United Kingdom and Dr. M. A. Linggood, Unilever Research, United Kingdom, respectively. The strains of *Pseudomonas aeruginosa*, *Bacillus subtilis,* and *Agrobacterium tumefaciens* were obtained from Food Microbiology Department, CFTRI, Mysore, India. (15). All test organisms were maintained on nutrient agar slants (Hi Media chemicals, India). Cultures of *S. aureus (*FRI 722)*, B. cereus* (F 4433), *E. coli* (D 21), *P. aerugenosa* (CFR 1704) were grown in brain heart infusion broth (Hi Media, India) for 18h at 37 ^{0}C and appropriate cell dilutions were prepared in 0.85 % NaCl to obtain counts of 10^2 and 10^3/ml (16). The respective bacterial counts were determined by surface plating on Baird-Parker agar for *S. aureus,* MacConkey agar for *E. coli* and Pseudomonas agar for *P. aeruginosa* (Hi Media, India). Cell suspensions of *Bacillus* species were prepared following the method of Rappaport and Goepfert (17) and cell dilutions were determined by surface plating on Polymyxin Pyruvate Egg yolk Mannitol Bromothymol blue agar (PEMBA) (Hi Media, India).

Determination of total phenolic compounds in SDG extracts

The concentration of total phenolic compounds in the extracts was determined according to the method of Taga *et al.,* (18) and expressed as caffeic acid equivalents. In brief, samples and standards were prepared in acidified (3 g/l HCl) methanol/water (60:40 v/v) and 100 µl of each were added separately to 2 ml of 2% Na2CO3. After 5 min, 100 µl of 50% Folin–Ciocalteu reagent was added and the mixture was allowed to stand at room temperature for 30 min. Absorbance was measured at 750 nm using spectrophotometer (Shimadzu 160A). The blank consisted of all reagents and solvents without sample or standard. The standard caffeic acid was prepared at concentrations of 10-100 µg/ml. The phenolic concentration was determined by comparison with the standards.

In vitro screening for antibacterial activity of SDG extracts

Agar-well diffusion assay

The antimicrobial activity was measured by agar well diffusion assay method (19). Extracts dissolved in ethanol (5 mg/ml) was used for the assay. About 75 µl of the sample was placed in the wells and allowed to diffuse for 2 h. Plates were incubated at 37 ^{o}C for 48 h and the activity was determined by measuring the distance of inhibition zones. Ethanol and DMSO alone were used as a control and amoxycillin as a positive control. The assay was carried out in triplicate.

Minimum inhibitory concentration (MIC)

The MIC was determined by the modified method developed by Dufour *et al*., (20) and Gary *et al.,* (21). Different concentrations (50 ppm to 300 ppm) of test sample and 100 µl of the bacterial suspension (10^5 CFU/ml) was placed aseptically in10 ml of nutrient broth and incubated for 24 h at 37 ^{o}C. The growth was observed both visually and by measuring O.D. at 600 nm at regular intervals followed by plating with nutrient agar. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. The sample tubes were maintained for each concentration of test sample and the readings were plotted against O.D at 600 nm as growth curves.

Statistical analysis

The data from three replicates were processed by one-way ANOVA using the least significant test to determine the level of significance at $P \leq 0.05$.

Results and Discussion

SDG contents of hull, endosperm and flour fractions of flaxseed

SDG contents were measured in different fractions of both verities of flaxseeds. The HPLC chromatograms showed the presence of SDG in all the fractions of flaxseed as one of the major lignan among the other lignans, which has shown maximum absorbance at 280 nm and the retention time for SDG was found to be 29-30 min as shown in Figures 1 and 3. In both the varieties, hull fractions showed higher SDG content (16.9 ± 1.25 and 12.5 ± 1.18 mg/g) followed by flour and endosperm fractions. The SDG content of hull and flour fractions were higher by 14 and 5-fold in LVF-01 variety and 20 and 8-fold higher content in GVF-03 variety respectively, when compared to endosperm fraction. The data are presented in the Table 1.

Table 1. Phenolic and SDG contents of hull, endosperm and flour fractions of LVF – 01 and GVF – 03 varieties.

Fractions	Flaxseed varieties	Total phenolics (mg/g)	SDG content (mg/g)
Hull	LVF-01	3.18 ± 0.56	16.9 ± 1.25
	GVF- 03	2.70 ± 0.74	12.5 ± 1.18
Endosperm	LVF-01	0.54 ± 0.08	1.2 ± 0.92
	GVF- 03	0.22 ± 0.02	0.6 ± 0.03
Flour	LVF-01	1.34 ± 0.65	5.8 ± 1.42
	GVF- 03	0.80 ± 0.02	4.6 ± 1.21

Phenolic contents of hull, endosperm and flour fractions of flaxseed

The content of phenolic compounds in all the fractions of both the flaxseed varieties were estimated (Table 1). The total phenolics of different fractions were found to be 3.18, 0.54 and 1.34 (LVF-01) and 2.70, 0.22 and 0.80 mg/g (GVF-03) in hull, endosperm and flour respectively. Both the varieties showed higher phenolic contents in hull fractions compared to that of the endosperm and flour fractions. The endosperm fraction had the least phenolic content in both the varieties. Total phenolics content in LVF-01 variety was 5.06 mg/g and 62, 11 and 27% was recovered in hull, endosperm and flour

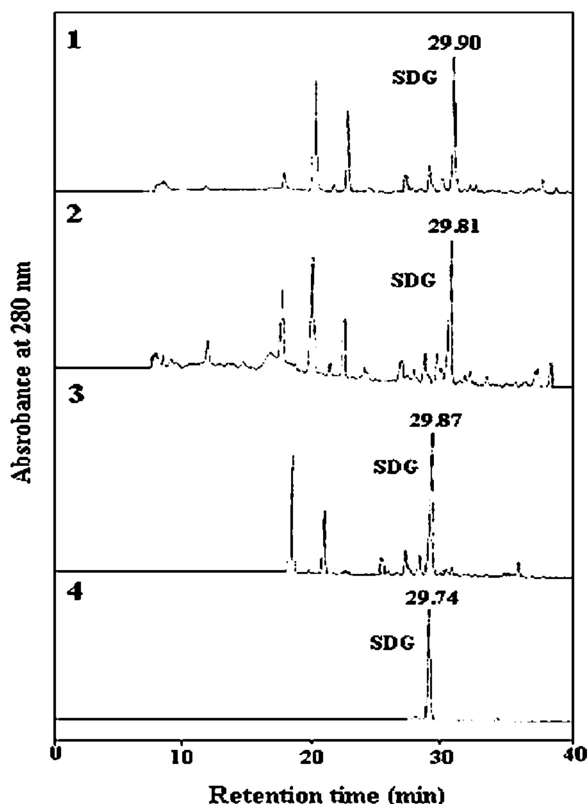

Fig. 2: HPLC chromatograms of SDG extracts of hull (1), endosperm (2), flour (3) and standard (4) of LVF-01 variety.

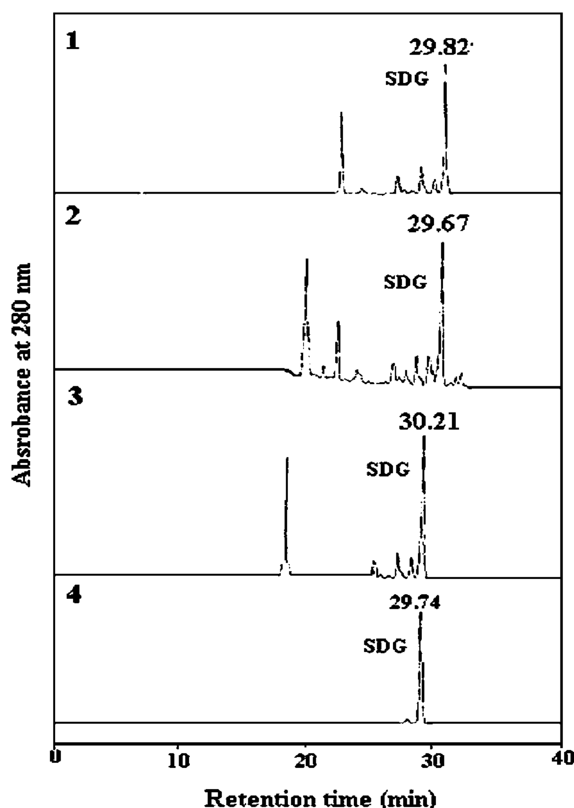

Fig. 3: HPLC chromatograms of SDG extracts of hull (1), endosperm (2), flour (3) and standard (4) of GVF-03 variety.

fractions respectively, whereas, the phenolics content in GVF-03 variety was 3.72 mg/g and 72, 6 and 22% was recovered in hull, endosperm and flour fractions respectively. When compared to GVF-03 variety, LVF-01 variety had more total phenolics content (36%). The results showed that hull fraction of LVF – 01 and GVF – 03 contained a higher amount of phenolics than the other two fractions.

Antibacterial properties of SDG extracts of hull, endosperm and flour fractions of flaxseed

The extracts of SDG from different fractions of both LVF-01 and GVF-03 were evaluated for their antibacterial activity as shown in Table. 2. The varieties, LVF-01 and GVF-03 were specifically selected for antibacterial properties, because of their wide cultivation and widespread use at lower levels as food substitutes in Northern Karnataka. The evaluation of the antibacterial activity of the SDG extracts agains bacteria was carried out by agar well diffusion assay method. The SDG of the flax seed fractions of both varieties exhibited antibacterial activity against all tested bacterial strains and showed various degrees of inhibition against them. The SDG from hull fractions of LVF-01 showed maximum

activity (31.5 mm) against *E. coli*, while it was minimum (9.7 mm) against *B. cereus*. Similarly, endosperm SDG exhibited maximum (14.87 mm) activity against *S. aureus* and minimum activity (3.13 mm) against *B. subtilis*. Flour fraction SDG exhibited maximum and minimum activity (22 and 6.83 mm) against *E. coli* and *B. cereus* respectively (Table 2). On the other hand, hull fraction of GVF-03 showed most pronounced activity with inhibition zones of 31.97 mm and minimum activity with 8.7 mm against *E. coli* and *B. cereus* respectively. Similarly, maximum and minimum activities (13.43 and 2.33 mm) for *S. aureus* and *B. subtilis* was observed by SDG from endosperm fraction and also flour-SDG showed maximum (21.57 mm) activity against *E. coli* and minimum activity (7.37 mm) against *B. cereus*.

Table 2. Antibacterial activity of SDG isolated from different verities (LVF- 01 and GVF -03) of flaxseed against bacteria.

Diameter of zone of inhibition (mm)						
Microorganisms	LVF - 01			GVF - 03		
	Hull	Endosperm	Flour	Hull	Endosperm	Flour
P. aeruginosa	24.7 ± 2.3	6.4 ± 0.4	14.0 ± 0.2	22.5± 1.4	4.4 ± 0.3	13.2 ± 1.6
S. aureus	31.5 ± 3.0	14.8 ± 1.0	18.3 ± 1.9	31.7 ± 2.1	13.4 ± 1.3	18.3 ± 2.3
B. subtilis	23.4 ± 1.9	3.1 ± 0.2	16.4 ±1.2	20.7 ± 1.3	2.3 ± 0.2	15.9 ± 1.8
A. tumefaciens	30.3 ± 2.0	10.4 ± 1.2	18.0 ± 1.7	31.2 ± 2.2	8.9 ± 1.0	17.0 ± 1.5
B. cereus	9.7 ± 1.3	7.8 ± 1.9	6.8 ±0.4	8.7 ± 1.3	7.6 ± 1.8	7.3 ± 0.9
E. coli	34.0 ± 2.8	14.0 ± 1.6	22.0 ± 1.6	31.9± 2.4	12.6 ± 1.5	21.5 ± 2.5

Each value represents mean of three different observations ± S.D.

MIC values for SDG extracts

The MIC values of SDG fractions of hull, endosperm and flour of LVF - 01 ranged from 100 to 300 ppm (Table 3). SDG fraction of hull was very effective against *E. coli* with MIC of 100 ppm and it also inhibited the growth of *S. aureus* and *A. tumefaciens* at 150 ppm. *P. aeruginosa* and *B. cereus* were completely inhibited by SDG fraction of hull at 200 and 300 ppm respectively. SDG extract of endosperm showed inhibitory activity against *S. aureus, A. tumefaciens* and *E. coli* with MIC of 250 ppm and *P. aeruginosa, B. cereus* and *B. subtilis* at 300 ppm. SDG extract of flour fraction showed inhibition against *E. coli* with 200 ppm and *S. aureus, A. tumefaciens* at 200 ppm.

The MIC values of SDG fractions of GVF-03 ranged from 150 to 300 ppm (Table 3). SDG extact of hull showed inhibition against *S. aureus* *A. tumefaciens* and *E. coli* with MIC of 150 ppm and also it inhibited the growth of *P. aeruginosa* *B. subtilis* at 200 ppm. SDG extract of endosperm fraction showed inhibitory activity against S. *aureus* and *E. coli* with 250 ppm. Similarly, in the case of SDG extract of flour fraction showed inhibitory activity against *E. coli*

at 200 ppm. *S. aureus, B. subtilis, B. cereus* were completely inhibited by SDG extract of flour fraction at 250 and *P. aeruginosa* with MIC of 300 ppm. All the SDG extracts of flaxseed fractions exhibited varied degrees of antibacterial activity. SDG extract of hull fraction of LVF-01 showed higher activity when compared to other fractions.

Table 3. MIC values for SDG extracts from different fractions of flaxseed against bacteria.

	MIC (ppm)					
	LVF - 01			GVF - 03		
Microorganisms	Hull	Endosperm	Flour	Hull	Endosperm	Flour
P. aeruginosa	200	300	300	200	350	300
S. aureus	150	250	250	150	250	250
B. subtilis	200	300	300	200	350	250
A. tumefaciens	150	250	250	150	300	300
B. cereus	300	300	300	300	300	250
E. coli	100	250	200	150	250	200

The present study evaluated the antibacterial properties of SDG isolated from different fractions such as hull, endosperm and flour of two Indian flax seed cultivars against important pathogenic bacteria. The SDG extract of hull of LVF-01 and GVF-03 showed higher activities, when compared to endosperm and flour fractions. SDG extracts of hull of both LVF-01 and GVF-03 showed inhibitory activity against *E. coli*, *S. aureus, A. tumefaciens* SDG extracts of endosperm fraction of LVF-01 and GVF-03 exhibited inhibitory activity against *S. aureus*, *A. tumefaciens* and *E. coli*. SDG isolated from flour fractions (LVF-01 and GVF-03) also showed inhibitory activity against *E. coli*. In general, among the investigated extracts of SDG, the extracts of hull of both varieties exhibited highest antibacterial activity than the other two fractions. The results show that the zone of inhibition is a practical approach for screening different concentrations of potential antimicrobial substances. The activities of the SDG extracts of different fractions of flaxseed against bacteria may be indicative to the broad spectrum antibiotic compounds. The differences in antibacterial activity among all the extracts may be correlated with varied quantity of bioactive compounds and phenolics in particular. At low concentration, phenolics are reported to affect enzyme activity, especially of those enzymes associated with energy production while at greater concentrations, they cause protein denaturation. In addition, effect of phenol and fatty acids on microbial growth could be the result of the ability of these compounds to alter microbial cell permeability, permitting the loss of macro-molecules from the interior and could also interact with membrane proteins causing a deformation in their structure and functionality as well as affecting cellular activity as reported by Mundt *et al.* (22).

The spectrum of activity of SDG isolated from different fractions of flaxseed of both

varieties were active against *E. coli*, and virtually showed less activity against *B. cereus* and *B. subtilis*. In some cases, all the three extracts of the same species had antimicrobial activity against the same microorganism. For instance, the three extracts of LVF-01 were active against *E. coli*. This possibly means that the compound responsible for the antimicrobial activity was present in each extract at a different concentration. Added to this, different results concerning the antibacterial activity might be due to different geographic sources of and types of flaxseed cultivars used. Thus, the difference in the antimicrobial activity of the isolated compounds against gram-positive and gram-negative bacteria may of our study regarding the antibacterial property of SDG extracts indicate that this could be used against the most common pathogens. However, the SDG extracts isolated from different fractions such as hull, endosperm and flour must be studied in animal models to determine their efficacy *in vivo* and possible toxicity, and to elucidate their mechanisms of action.

Conculsion

In the present study, it is concluded that the SDG extracts of hull, endosperm and flour from two Indian flaxseed verities are having potential antibacterial activity against pathogens. Among the fractions, the hull fractions were showing higher antibacterial activity when compared to other fractions. The antibacterial effect of SDG of hull and endosperm of flaxseed against clinically important pathogenic bacteria can be a preferred supplement to its known health benefits as antibacterial agents and usage in food system. Further investigations are in progress to study the biological activities of these fractions. Therefore, the different fractions of flaxseed may be recognized as a contributing factor in the preparation of such a type of human health foods as well as others. Further more, careful investigations are required to elucidate the mechanism(s) of action of these compounds. The presence of significant amount of SDG and other lignans in flaxseed, may therefore explain the frequent use of it in a variety of Indian medicinal preparations.

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Callus Induction and Antimicrobial Activity of Seed and Callus Extracts of *Clitoria ternatea* L.

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Abstract

Callus induction from leaf derived explant of *C. ternatea* was achieved with 100% frequency on Murashige and Skoog's basal medium fortified with 2 µM 2 4- D and 18 µM kinetin. Callus grew in size and weight till 6th week in culture as measured by an increase in both fresh and dry weights. Aqueous extracts of both seed and callus were prepared for evaluating the antimicrobial activity against selected pathogenic fungi and bacteria using the agar well diffusion technique. Seeds and leaf delivered calli of *C. ternatea* were extracted using standardized laboratory protocol. The seed extract of *C. ternatea* showed maximum zone of inhibition (22 ± 0.5 mm) against *Escherichia coli* (NCIM 2645) at 0.75 mg concentration and minimum (14 ± 1.0 mm) with *Micrococcus flavus* (NCIM 2376). The callus extract showed maximum zone of inhibition (16 ± 2.0 mm) against *Salmonella typhi*, the minimum was against *Escherichia coli* (NCIM 2645) and *Staphylococcus aureus* (12 ± 1.0 mm and 12 ± 0.9 mm, respectively). The seed extract of *C. ternatea* showed strong antifungal activity on all the tested fungi but the callus extract exhibited marginal antifungal activity.

Keywords: *Clitoria ternatea*; seed, callus, antimicrobial activity.

Introduction

Use of plants as a source of medicine has been inherited and is an important component of the indigenous health care system. The WHO estimates that more than 80% of the world's population rely either solely or largely on traditional remedies for health care (1). Approximately 20% of the plants found in the world have been submitted to pharmacological or biological tests (2). In India, about 2500 species are used for medicinal purposes, and about 90% of the medicinal plants provide raw material for the herbal pharmaceuticals, which are collected from the wild habitats (3). The systemic screening of antimicrobial agents from plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant pathogenic bacteria and fungi (4). *Clitoria ternatea* L. (butterfly pea in English) belongs to the family Fabaceae and subfamily Papilionaceae, is a herbaceous perennial legume valued for its forage and medicinal importance (5). The plant has been adopted in the traditional Indian system of medicine due to its multiple pharmaceutical applications (6). The active constituent(s) include tannins, resins, starch, kaempferol and its glucoside-clitorin, taraxerol, taraxerone and a lactone aparajitin. It has been recommended as a rejuvenating brain tonic having anxiolytic, antidepressant, anticonvulsant, and anti-stress properties and is believed to promote memory and intelligence (7 - 9). The whole plants and seed

extracts are useful in stomatitis piles, sterility in female, hematemesis, insomnia, epilepsy, psychosis, leucorrhea and polyurea (10). The seeds are purgative, cathartic, and useful in visceralgia. Besides, it contains antifungal protein (finotin) which has been shown to be homologous to plant defensins (11).

In the present paper, we report callus induction and antimicrobial activity of callus and seed extracts of *C. ternatea*.

Materials and Methods

Pods of mature seeds of high yielding cultivar of butterfly pea were locally collected and authenticated by an expert taxonomist. The seeds were stored in sealed containers at room temperature and used throughout the year. When required they were grown in plastic pots containing black cotton soil, irrigated with tap water and maintained in sunlight in the laboratory at room temperature. Six different bacterial strains namely *Bacillus subtilis* (NCIM 2010), *Escherichia coli* (NCIM 2645), *Micrococcus flavus* (NCIM 2376), *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhii* and four different fungal strains namely *Aspergillus flavus* (NCIM 519), *Aspergillus ochraceous* (NCIM 1140), *Rhizopus oryzae* (NCIM 877) and *Aspergillus niger* were obtained from collections maintained at School of Life Sciences, North Maharashtra University, Jalgaon (MS).

Murashige and Skoog's (MS) medium (13) comprising of MS basal salts and MS vitamins and supplemented with 3% (w/v) sucrose, gelled with 0.8% (w/v) agar was selected for carrying out all the experiments. The pH of medium was adjusted to 5.6 – 5.8, using 0.1 N NaOH or HCl prior to addition of agar and sterilization at 1.05 Kg. cm^{-2} pressure and 121^0 C for 20 minutes. Thermo-labile constituents like vitamins and phyto-hormones were filter sterilized through 0.2µ membrane before addition to the sterilized medium.

For callus induction, leaves from 20-days-old laboratory grown plants were cut and surface sterilized by a laboratory standardized protocol (14) before culturing. Each leaf explant was cultured on 20 mL of MS medium enriched with various concentrations and combinations of 2,4-D and kinetin in glass culture tubes. Explants were positioned with their dorsal surfaces touching the medium. All the operations were carried out in a laminar flow unit (Kirloskar Electrodyne, Bhosari, Pune) under sterile conditions.

Preparation of extracts

Ten grams seeds of *C. ternatea* were surface sterilized initially in 70% (v/v) ethanol for 3 minutes followed by 4% (v/v) NaCl solution for 10 minutes and rinsed with sterile distilled water (5 to 6 times). The disinfected seeds were left in sterile distilled water overnight to facilitate maceration. The imbibed seeds were then macerated in 50 mL of sterile distilled water with the help of mortar and pestle. The macerated solution was filtered through cheesecloth to get rid of the seed debris. The filtrate was then centrifuged at 4^0 C in centrifuge tubes at 13000 x g for 30 minutes. The supernatant was filtered through 0.22µ membrane. The supernatant was used as a source of bioactive metabolite and bioassays were performed with it. Similarly, ten grams dried powder of leaf derived callus (6 – 7 weeks old) of *C. ternatea,* was first soaked in 50 mL sterile distilled water and filtered through cheesecloth. The filtrate was then centrifuge at 4^0 C in centrifuge tubes at 13000 x g for 30 minutes. The supernatant was filtered through 0.22µ membranes and used for antimicrobial bioassays. The total protein in the extracts was estimated by Lowry's method with BSA as an internal standard (15).

Antimicrobial activity

The extracts were checked for antimicrobial activity using the agar well diffusion technique

(16 - 17). A 100 μL aliquot of each test microorganism, inoculum size (10^6 cfu/mL for bacteria and 2 X 10^5 spores/mL for fungi) was spread on to sterile nutrient agar plates and Czapekdox agar plates, respectively with the help of sterile glass spreader so as to achieve a confluent growth. The plates were allowed to dry and a sterile cork borer of diameter 6.0 mm was used to bore wells in the agar plates. Different concentrations of crude extracts from seeds as well as callus in 50 μL aliquots were loaded in well. Amipicillin (25 μg/disc) and nystatin (100 units/disc) were used as standard antimicrobial antibiotics. The plates were allowed to stand for 1 h or more for diffusion to take place. Each test was carried out in triplicate. The plates were incubated at 37^0 C for 24 h for bacteria and 28^0 C and 48 h for fungi. Inhibition of microbial growth was determined by measuring the diameter of zones of inhibition in mm by an antibiotic zone scale (Hi Media, Mumbai).

Results and Discussion

Murashige and Skoog's (MS) medium lacking plant growth regulators failed to produce callus. Callus initiation started from the cut ends of the explant (Fig. 1a) and covered the whole surface of the leaf disc within 4 week. A 100% frequency was achieved on MS basal medium fortified with 2,4-D and kinetin at 2 and 18 μM concentration, respectively (Table 1). The fresh and dry weights of callus were taken as an index of growth. Accordingly, it was observed that the doubling rate of cells was highest up to sixth week of culturing after which it showed a declining trend (Table 2). Initially, callus was soft and brownish in colour and after 3 to 4 weeks it became compact and greenish in colour (Fig. 1b). The protein yield from callus and seed extract was 3.25 and 9.06 gm%, respectively.

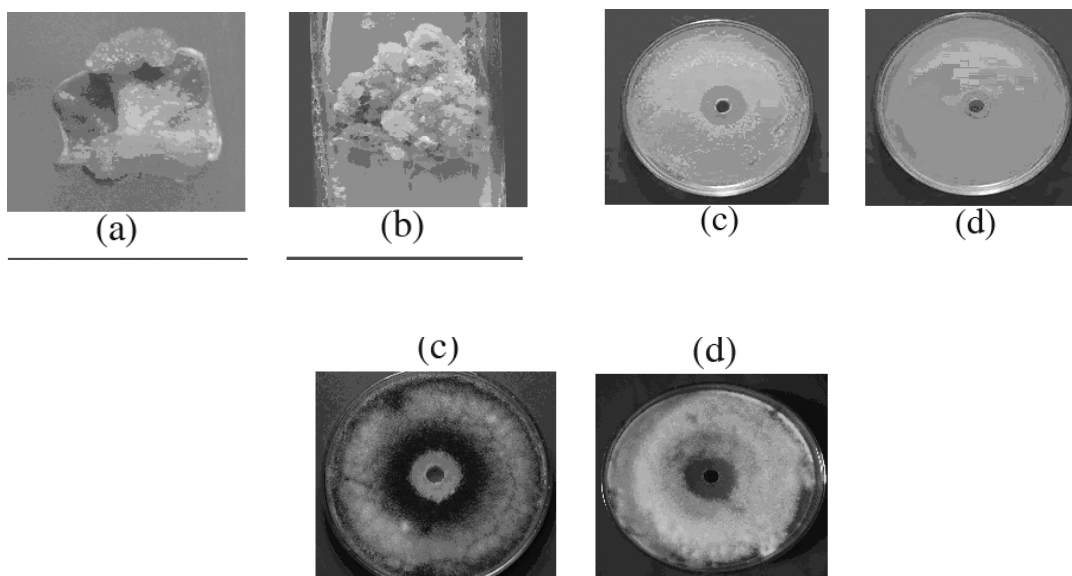

Fig. 1: (a) Initiation of callus induction from leaf explant of *C. ternatea*, **(b)** A 6 week old leaf derived callus, **(c)** Antibacterial activity of seed extract against *E. coli*, **(d)** Antibacterial activity of callus extract against *S. typhi,* **(e)** & **(f)** Antifungal activity of seed extract against *A. niger* and *R. oryzae*.

Table 1: Effects of different concentrations of 2,4-D and kinetin added to the MS medium on callus induction from leaf explants of *Clitoria ternatea*.

Phytohormones (μM)		Frequency of callus induction (%)
2,4-D	Kinetin	
0	0	00
2	2	60
2	6	60
2	10	80
2	14	80
2	18	100
2	20	60
2	2	20
6	2	40
10	2	10
14	2	10
18	2	10
20	2	20

Data were taken after 4 weeks of culture.
Each experiment was done in triplicate and number of explants per replicate was 25.
The values are means of three replicates.

Table 2: Growth profile of leaf derived callus of *Clitoria ternatea* as a function of time in culture

Age of callus (weeks)	Weight (g)	
	Fresh	Dry
0	00	00
2	0.03	0.003
3	0.29	0.025
4	0.80	0.230
5	1.20	0.595
6	1.80	0.840
7	2.00	0.945
8	1.30	0.723
9	1.03	0.531
10	0.93	0.255
12	0.89	0.215

MS basal medium fortified with 2 μM 2,4-D + 18 μM kinetin was used.

Antimicrobial activity

The antibacterial activity of the crude extracts of seed and callus is shown in Table 3. The crude extract from seeds of *C. ternatea* showed maximum zone of inhibition (22 ± 0.5 mm) against *Escherichia coli* (NCIM 2645) at 0.75 mg concentration (Fig. 1c) and minimum with *Micrococcus flavus* (NCIM 2376) of 14 ± 1 mm. The callus extract showed maximum zone of inhibition (16 ± 2 mm) against *S. typhi* (Fig. 1d) while the lowest with *Escherichia coli* (NCIM 2645) and *Staphylococcus aureus* (12 ± 1 mm and 12 ± 0.9 mm, respectively). The crude extracts showed activity against all tested microorganism in the present study. Of the different concentrations tested, 0.75 mg concentration exhibited maximum inhibition against test bacterial strains. Ampicillin (25 µg/disc) was taken as standard antibacterial agent. The antifungal activity of the crude extract of seed and callus are shown in Table 4. The crude extract from seeds of *C. ternatea* showed strong antifungal activity on the test fungus *Aspergillus niger* and *Aspergillus ochraceous* (NCIM 1140) (Fig 1e and 1f) followed by other organisms. The activity of callus extract against test fungi was comparatively less.

Table 3: Antibacterial activity of seed and callus extracts of *C. ternatea*

Microorganism	Zone of inhibition (mm)						Std. (A^{25})
	Seed extract (mg)			Callus extract (mg)			
	0.5	0.75	1.0	0.5	0.75	1.0	
***Bacillus subtitis* (NCIM 2010)**	21 ± 1.0	21 ± 0.0	20 ± 0.6	13 ± 0.5	14 ± 0.9	11 ± 1.0	16
***Escherichia coli* (NCIM 2645)**	19 ± 1.0	22 ± 0.5	23 ± 0.9	10 ± 1.0	12 ± 1.0	12 ± 1.0	16
***Micrococcus flavus* (NCIM 2376)**	13 ± 0.5	14 ± 1.0	13 ± 0.9	11 ± 1.0	13 ± 0.5	13 ± 1.0	>10
Staphylococcus aureus	12 ± 1.0	15 ± 0.5	14 ± 1.0	10 ± 0.9	12 ± 0.9	11 ± 1.0	10
Pseudomonas aeruginosa	13 ± 0.5	15 ± 0.0	15 ± 0.9	12 ± 1.0	15 ± 1.0	10 ± 1.5	>10
Salmonella typhi	13 ± 0.0	15 ± 1.0	16 ± 1.0	10 ± 1.0	16 ± 1.0	12 ± 1.7	>10

All the zones in 'mm' and each value represent an average of three replications ± SD
Diameter of the well was 6 mm
Standard antibiotic used was ampicillin (25 µg)

Till date, over 600 plants have been reported for their antifungal properties. However, only a few of them have been explored for the active component (4). Antifungal property of *C. ternatea*, due to presence of a small molecular weight, cystein rich protein, finotin, from seeds (18) and a flavanoid in the leaf extract (5) has been demonstrated. *C. ternatea is* also considered to be a promising and safe alternative natural product based antifungal agent for future generations (5). Isolation of active principle from the cultured cells *in-vitro* offers an added advantage to understand and manipulate biosynthetic pathway for enhancing the yield and exploitation for commercial applications.

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Table 4: Antifungal activity of seed and callus extracts of *C. ternatea*

Microorganism	Zone of inhibition (mm)										Std. (A^{25})
	Seed extract (mg)					Callus extract (mg)					
	0.5	0.75	1.0	1.5	2.0	0.5	0.75	1.0	1.5	2.0	
Aspergillus flavus NCIM 519	13 ± 0.0	15 ± 1.2	18 ± 0.0	13 ± 0.0	15 ± 0.0	-	-	-	-	-	16
Aspergillus Ochraceous NCIM 1140	15 ± 0.0	20 ± 1.0	22 ± 1.7	24 ± 0.0	23 ± 1.0	-	-	T	T	11 ± 1.0	17
Rhizopus Oryzae NCIM 877	15 ± 1.0	18 ± 0.6	19 ± 0.0	21 ± 1.0	20 ± 1.2	-	-	-	-	-	18
Aspergillus niger	16 ± 2.0	19 ± 1.0	22 ± 0.0	24 ± 1.0	25 ± 1.0	-	-	T	11 ± 1.0	12 ± 1.0	17

All the zones in 'mm' and each value represent an average of three replications ± SD
. Diameter of the well was 6 mm
Standard antibiotic used was Nystatin (100 units)

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High Yield Expression of Human Recombinant PTH (1-34)

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Abstract

Human parathyroid hormone (hPTH), synthesized in the parathyroid gland as a linear peptide, is one of the key regulatory molecules in calcium homeostasis and bone resorption. The N terminus region of hPTH (1-34) is a functionally important part of the molecule that is sufficient and necessary for potently executing most of the hormonal actions. Therefore, hPTH (1-34) is considered to be an attractive therapeutic agent in the treatment of osteoporosis. Here, we describe a high yield expression and purification method for the production of hPTH (1-34) from Escherichia coli. The hPTH (1-34) was expressed as a fusion protein in soluble form, and found to be more than 25% of total proteins. The fusion protein was first purified by GST affinity chromatography and, after cleavage of GST with Factor Xa, the peptide (hPTH 1-34) was further purified by reversed phase Source-30™ chromatography. This double purification strategy produced 30mg/l of hPTH (1-34) with purity = 98%. The identity of the purified peptide was confirmed by mass spectrometry and N-terminal sequencing analysis. The biological activity of the peptide was confirmed in the rat osteogenic cell line UMR-106 by measuring cAMP levels, which were identical to hPTH standards, indicating that purified rhPTH (1-34) has full biological activity.

Key words: Factor Xa; Glutathione S transferase; Protein purification, Recombinant human parathyroid hormone.

Introduction

Parathyroid hormone (PTH) or parathormone is a naturally occurring peptide hormone secreted by the parathyroid glands. PTH is essentially the central endocrine regulator of calcium and phosphorous in the extracellular fluid. The hormonal action of PTH is effected through its binding to cell surface membrane receptors. The target tissues expressing PTH receptors are predominantly found in bone, kidney and intestine.

In humans, PTH is synthesized as a 115-amino-acid precursor polypeptide and undergoes maturation in the endoplasmic reticulum and Golgi apparatus. After processing it is secreted as an 84-amino-acid peptide (1). Interestingly, compared with the full 84 amino acid peptide, the processed peptide fragment consisting of amino acids 1-34 appears to contain all of the information necessary for executing the full biological activity obtained with the full 84-amino-acid peptide. In the original chemical synthesis of PTH, the phenylalanine residue at position 34 was chosen as the amino acid to couple to the solid support resin (2,3,4,5,6 and 7); virtually all studies on PTH for the past three decades have used this synthetic fragment or an analog of it.

PTH regulates mineral ion metabolism and bone turnover by activating specific receptors located on osteoblastic and renal tubular cells. The molecular outcomes of PTH interaction with its receptors have been extensively investigated. In these tissues PTH stimulates multiple intracellular signals including cAMP (3,4,5,6,7 and 8), calcium and phosphorus (9,10,11,12,13,14,and 15).

PTH binding results in receptor activation and stimulation of the adenylate cyclase complex, leading to the accumulation of intracellular cAMP, which activates protein kinase A that, in turn, phosphorylates key regulatory proteins. This altered phosphorylation status of the regulatory molecules presumably mediates the biological actions of PTH in its target tissues. The biological activity of the purified rhPTH (1-34) was assayed by its ability to stimulate renal adenylate cyclase. Renal adenylate cyclase was assessed by measuring the stimulation and accumulation of cAMP in the rat osteosarcoma cell line UMR-106.

Previous reports on the expression of biologically active peptides in Escherichia coli (E. coli) have indicated low protein yield, probably due to rapid intracellular degradation of the peptide, and the difficulty in purification of the peptide from endogenous contaminating proteins and peptides. Several groups have recently reported the expression of fusion proteins containing either the 1-84 or 1-34 form of PTH in E. coli. Expression from the E. coli lac promoter yielded not more than 500μg of immunoreactive-protein/l of culture (16,17). Gardella et al (18) have expressed hPTH (1-84) as a factor Xa (FXa) cleavable fusion protein with human growth hormone. Intact hPTH could be purified following FXa cleavage at levels of 1.5-3mg/l of original cell culture. Kareem et al (19) describe similar results using protein A as a fusion protein partner. Wingerder et al (20) reported improved yields of PTH using an acid cleavable fusion protein expression system. This system produced up to 250mg of fusion protein / l of culture, after treatment with formic acid the final protein yield was 3-5mg of [pro'] PTH. Olstad et al (21) reported expression of hPTH as a result of the union with yeast mating protein. That expression system resulted in the secretion of hPTH into the media and the peptide was found to be o-glycosylated, but an overall yield for the peptide in this system was not given.

Oldenburg et al (22) reported high yield production of hPTH (1-34) using a gene polymerization strategy. The PTH gene polymerization contains up to 8 copies of the gene, each separated by a cleavable linker. The monomeric rPTH (1-34) is released from the polymer by chemical cleavage with cyanogen bromide. Peptides produced through this methodology will also contain a homoserine/ homoserine lactone residue at the carboxyl terminus. Recognizing the potential of hPTH (1-34) and the limitations of the currently available strategies, we sought to develop an efficient method to produce recombinant hPTH (1-34) in high yield with high purity and activity for application on an industrial scale.

Materials and Methods

Material

E. coli host cells (Rosetta) were obtained from Novagen (Darmstadt, Germany). Plasmid (pGEX-2T), Source-30™ reversed phase chromatography matrix and GST affinity matrix columns were purchased from Amersham Biosciences (Buckinghamshire, England). Ultrafiltration membrane cassettes were purchased from Sartorious (Goettingen, Germany). The BioLogic DuoFlow chromatography system used for column

purification was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The rat osteosarcoma cells UMR-106 (ATCC# CRL-1661) were obtained from American type culture collection (Manassas, Virginia, USA) and the hPTH (1-34) standard Forteo was purchased from Eli Lilly (Indiana, USA). FXa was prepared in-house by isolation from bovine plasma and activation by a protease from Russell's viper venom.

cDNA synthesis, cloning and expression of rhPTH (1-34)

Poly(A) selected RNA was isolated from human parathyroid adenomas immediately after surgery. The tissue was homogenized in RNA extraction buffer (500µl) containing 4M guanidium thiocyanate, 25mM sodium citrate (pH 7.0), 0.5% sacrosyl and 0.1M 2-mercaptoethanol at 4°C and disrupted for 1 min with a Polytron tissue homogenizer. 50µl of 2M sodium acetate, 500µl water saturated phenol and 200µl chloroform:isoamyl alcohol (49:1) were added to the lysate. The aqueous phase was collected after centrifugation at 10,000 rpm for 10 min. RNA was precipitated by the addition of 2.5 volumes of isopropanol and incubated at -70°C for 1h. The RNA pellet was washed with 70% ethanol and resuspended in 20µl of water and stored at -70°C until use. All solutions were made up in DEPC-treated distilled water. RNA was isolated, and poly(A) mRNA was prepared by oligo(dT) chromatography as described previously (23).

hPTH(1-34) cDNA was synthesized from mRNA by using the following gene specific reverse primer: 5'-AAA ATT GTG CAC ATC CTG CAG -3'.

Specific primers were designed for synthesizing hPTH (1-34) having the FXa cleavage site bearing the sequence 5‘ - CC GGA TCC ATC GAA GGT CGT TCT GTT TCT GAA ATC -3'as a forward primer and 5‘ - CC GAA TTC TCA AAA ATT GTG CAC -3' as a reverse primer.

The PCR amplified product hPTH (1-34) with FXa site and the vector pGEX-2T was restriction digested with BamH1 and EcoR1, and the insert was ligated into the vector to generate recombinant plasmid (pGEX-2T/hPTH (1-34)).

E. coli strain Rosetta (DE3) was transformed with recombinant plasmid containing the hPTH(1-34) gene. The expression of the hPTH (1-34) gene was driven by a Tac promoter, which can be regulated by inducing the culture with isopropyl beta-D-thiogalactopyranoside (IPTG), thereby allowing a high level of expression of the hPTH (1-34) gene as a fusion tag to GST.

High level expression and cell harvesting

The high-density fermentation of recombinant bacterial E. coli (pGEX-2T/PTH (1-34)) was carried out with a 5-l fermentor by fed-batch cultivation. 4.2l of sterile LB media were inoculated with 100ml of seed innoculum and supplemented with 2% dextrose. During the batch fermentation, the temperature and pH were maintained at 37^0C and 7.0, respectively. The dissolved oxygen level was maintained at 30-40% by using air or pure oxygen and the speed was maintained at 600 rpm. After the optical density (OD_{600}) reached 60, a final concentration of 1mM IPTG was added to induce the expression of fusion protein over 3h. After 3h, the cells were harvested by centrifuging at 4000rpm for 10 min at 4^0C and frozen at -70^0C. The expression was checked by running the samples on SDS-PAGE gels and staining with CBB stain (Fig. 1).

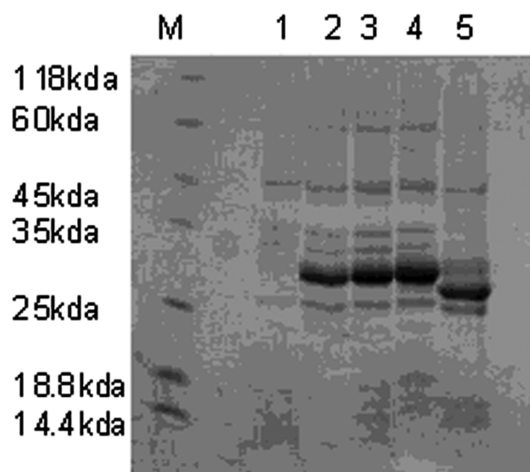

Fig. 1: Expression analysis of pGEX-PTH(1-34): The E. coli BL21 Rosetta cells were transformed with recombinant plasmid. The Transformed cells were induced with 0.5mM IPTG for 3h. The samples were collected every hour and lysed in lysis buffer and subjected to SDS-PAGE analysis. Lane M-marker, 1-4: induced samples after 0, 1, 2 and 3h, respectively. Lane5: vector alone.

Cell lysis and fusion protein isolation

The frozen pellet (105g) was thawed on ice and resuspended in 200ml of 1x PBS. The pellet was homogenized to get a suspension. The homogenate was lysed by using the bead-beater (Bio-Spec). Beads of 0.1mm size were washed with 1x PBS and added to the homogenate. Cell disruption was completed after 10 beating cycles, with each cycle 1min on, 1min off. After centrifugation the beads were removed from the suspension. Triton X-100 (1% final) was added and mixed gently by stirring for 30min at room temperature to aid solubilization of fusion protein. The solution was centrifuged at 18,000 rpm for 30 min at 4^0C in a Sorval RC-6 with an SV-800 rotor.

The clear solution was loaded on to the 200ml glutathione-sepharose 4B affinity column pre-equilibrated with 1x PBS (column bed height, 8cm, and diameter, 6cm). The column was washed with 4 bed volumes of 1x PBS to remove the unbound proteins. The bound fusion protein (GST-PTH (1-34)) was eluted with 600ml of 5mM reduced glutathione in 50mM Tris/HCl, pH 8.0. Fractions of 100ml were collected. The purity of the fusion protein was analyzed via SDS-PAGE gels. The yield of fusion protein was calculated by taking the OD of the pure fractions at 280 nm (OD_{280} 1 = 0.5mg/ml).

Cleavage of fusion protein

The pure fractions of fusion protein were pooled and dialyzed into 50mM Tris/HCl, pH 8.0, 150mM NaCl and 2mM $CaCl_2$, to remove the reduced glutathione. The dialyzed fused GST-hPTH protein was cleaved with FXa (1:200 w/w enzyme to fusion protein ratio) at 22^0 C for 2h. FXa was purified from bovine blood in our laboratory. To collect the hPTH (1-34), the digested sample was reloaded onto the GST affinity column and the unbound protein was collected.

Purification of hPTH(1-34) by HPLC

The cleaved hPTH (1-34) was further purified using reversed phase column chromatography. To the sample 0.1% TFA was added, followed by loading onto the Source-30 RP matrix packed into a 50ml column. The column was pre-equilibrated with 0.1% TFA.

The pure peptide was eluted with a linear gradient of acetonitrile (acetonitrile 0%/0.1%TFA - acetonitrile 70%/0.1%TFA). The pure fractions were analysed by SDS-PAGE. The peptide was then lyophilized to remove the acetonitrile. The purity of the peptide was analyzed by running the sample on an analytical C18 column. The molecular weight and amino acid sequence were confirmed by mass spectra and N-terminal sequencing.

Biological activity

The biological activity of the purified rhPTH (1-34) was assayed by its ability to stimulate renal

adenylate cyclase. Renal adenylate cyclase was assessed by measuring the stimulation and accumulation of cAMP in the rat osteosarcoma cell line UMR-106.

UMR-106 cells (ATCC# CRL-1661) were obtained from American type culture collection. The cells were maintained in a humidified 5% CO_2 atmosphere and were cultured in DMEM medium supplemented with 10% fetal bovine serum. For the assay, the cells were sub-cultured into a 24-well plate in regular medium to a confluence of 70-80%. The cells were then treated with different concentrations of rhPTH (1-34) and standard PTH for 1h at 37^0C in the absence of serum. After incubation with PTH, the cells were washed with 1XPBS and lysed in 1ml of 0.1N HCL on ice. The accumulated cAMP was determined by an indirect cAMP enzyme immunoassay kit from Assay Designs

The principle of the immunoassay kit (Assay Designs' Correlate™ EIA Direct cyclic AMP kit) is, briefly, a competitive immunoassay for the quantitative determination of cAMP in samples treated with 0.1M HCl. The kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the standard or sample or an alkaline phosphatase molecule that has cAMP covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation time the enzyme reaction is stopped and the yellow color generated read on a micro plate reader at 405 nm. The intensity of the yellow color is inversely proportional to the concentration of cAMP in either the standards or samples. The measured OD is used to calculate the concentration of cAMP.

Results and Discussion

Expression plasmid construction

In the preparation of polypeptides by genetic engineering, expression via fusion protein has often been used to: (i) prevent degradation of the polypeptides by the proteases in the host, (ii) increase the expression yield, and (iii) design the lead sequence in the fusion protein as a tag for use in affinity purification. The internal protease sensitive domains make hPTH susceptible to degradation and inactivation (24,25). The major proteolytic cleavage sites are after Val^{21} (26). In this paper we have described a high yield system for producing hPTH (1-34) from bacteria.

The pGEX-2T vector was used to construct an expression plasmid. The hormone is expressed from the plasmid pGEX-2T as a cleavable fusion protein. The cDNA was placed downstream from Tac promoter. An extra amino acid sequence containing the FXa cleavage site was placed at the N-terminus of PTH (1-34) to allow cleavage with FXa, which eliminates the fragment of extra amino acids. The hPTH cDNA coding sequence was inserted into the BamH1 and EcoR1 site of the plasmid.

The GST-hPTH fusion protein was overproduced in E. coli cells transformed with pGEX-2T-hPTH plasmid in an inducer dependent manner. The high level of GST/PTH fusion protein obtained may also be partly due to increased mRNA or protein stability of the hybrid sequences relative to the native PTH sequence alone. Observations suggested that expression of hPTH directly in E. coli led to low product levels and protein degradation due to mRNA and protein instability (16,17).

Induction of Tac promoter by the addition of IPTG to the cell culture resulted in accumulation of fusion protein to the extent that it was the most abundant protein detected by SDS gel analysis of the whole cell lysates. In the induced cells a predominant band migrating with a molecular mass of approximately 30kDa was observed, which was not detected in the un-induced cells (Fig. 1). The percentage of the

expressed protein versus total protein was detected as approximately 25% when quantified by densitometry. The total wet weight of the cell pellet was 20-25g/l culture.

Purification of fusion protein and cleavage

Like many other proteins that are overproduced in E. coli cells, the GST-PTH fusion protein was secreted into the cytosol. The fusion protein was solubilized and extracted by lysing and solubilizing with 1% Triton X-100. The fusion protein was purified over immobilized glutathione, which yielded over 85% pure fusion protein (Fig. 2). The yield of the fusion protein was 350-400 mg/l culture. After dialysis against FXa cleavage buffer, the fusion protein was cleaved with FXa. This yielded two cleavage products that migrated on SDS gels with molecular masses of 26 and 4kDa, corresponding to the GST and PTH (1-34) fragments, respectively (Fig. 3).

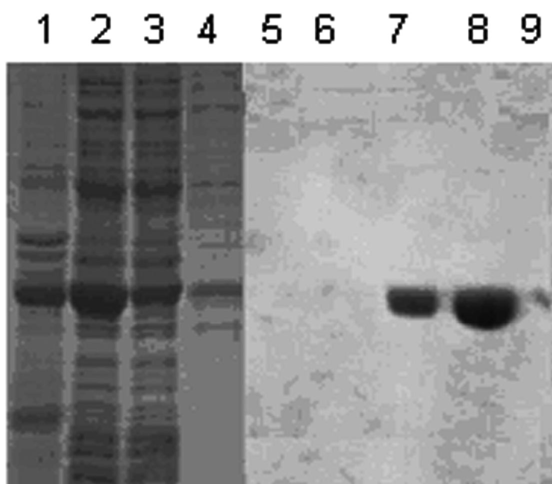

Fig. 2: SDS-PAGE analysis of processed samples: The cell pellet was lysed by using a bead beater and solubilized with Triton X-100, and the fusion protein was purified over GST matrix. The bound fusion protein was eluted with 5mm reduced glutathione. The fractions were analyzed by SDS-PAGE. Lane 1: pellet after solubilization. Lane 2: supernatent. Lane 3: unbound. Lanes 4 & 5 are washes and lanes 6-9 are eluted fractions.

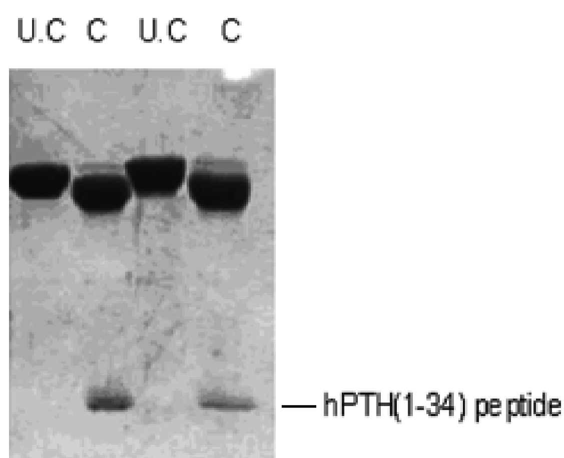

Fig. 3: Analysis of fusion protein cleavage with factor Xa:
The eluted fusion GST/PTH (1-34) protein was dialyzed into factor Xa cleavage buffer. The fusion protein was then cleaved with factor Xa for 2 and 4h at 22^{0}C with stirring. Lane UC: uncut sample, C: cut sample. Samples were subjected to SDS-PAGE to determine the percentage of cleavage.

Purification of hPTH(1-34) by HPL:

The cleaved product was purified over RPC Source-30 matrix. The peptide was eluted using a linear gradient of 24-28% acetonitrile (Fig. 4). After reverse phase chromatography, the rhPTH (1-34) was estimated to be greater than 98% pure. The results of an analytical run (Fig. 5), SDS gel electrophoresis (Fig. 6) and partial N-terminal amino acid sequence analyses indicated that the recombinant hPTH product had the expected structure and that no major contaminating proteins were present. The recombinant hormone eluted on HPLC as a single peak and had the same retention time as synthetic hPTH (1-34). The yield of the pure peptide was 25-30 mg/l culture pellet (Table 1).

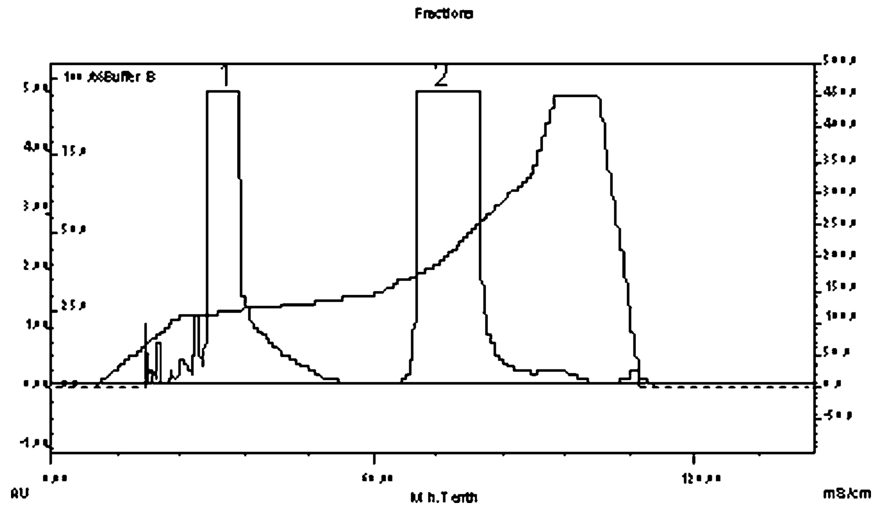

Fig. 4: Reversed phase HPLC purification of PTH(1-34):
After cleaving the fusion protein with factor Xa, the digest sample was reloaded onto a GST affinity column and the unbound protein was collected. The collected unbound protein was fractionated on a Source-30 column equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile. The pure fractions of proteins were collected and analyzed. The pure peptide peak (peak 1), which eluted at 25-28% acetonitrile gradient, was collected.

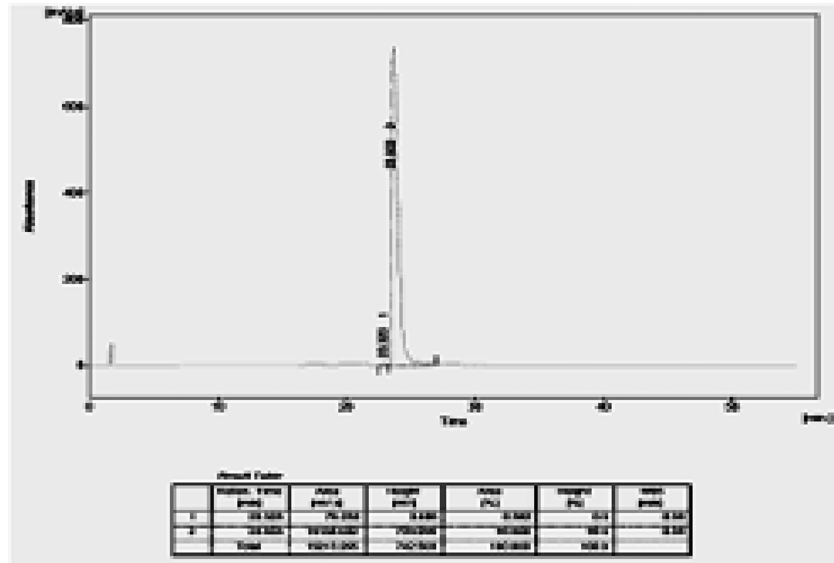

Fig. 5: Analytical HPLC profile of purified rhPTH(1-34):
The purity of the PTH 1-34 peptide was analyzed by loading the sample onto a C18 RP-HPLC column and eluting with a linear gradient of acetonotrile. The peptide was eluted at 23% acetonitrile. The purity of the peptide was estimated to be more than 98%.

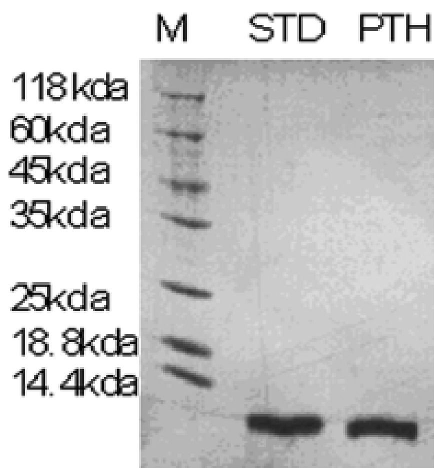

Fig. 6: SDS-PAGE analysis of pure PTH(1-34): The purified peptide was run on SDS-PAGE and visualized by the silver staining method. Both the standard peptide and rhPTH(1-34) migrated in the same manner and were at least 98% pure.

Table 1: Yields and purities achieved at each purification step.

Purification Step	Total proteins (mg)	Fusionprotein/ PTH(1-34) (mg)	Step yield(%)	Purity (%)
Whole cells	10500	2650*	100	25
Cell Lysis	4975	1990*	75	40
Affinity column	1880	1600*	80	85
Fxa digestion	206	175**	80	85
Affinity column	170	161**	92	95
RP-HPLC	148	147**	91	99

* Corresponds to Fusion protein.
** Corresponds to rhPTH (1-34).

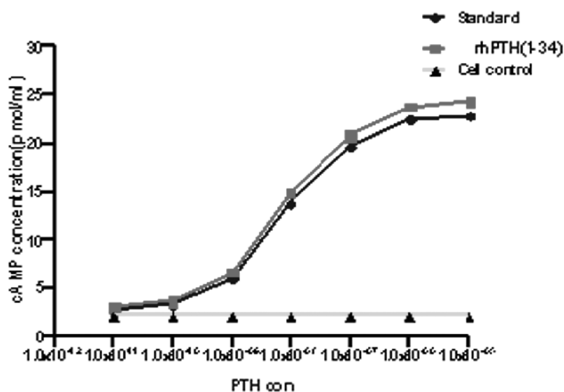

Fig. 7: Biological activity of rhPTH(1-34): The biological activity of the peptide was assessed against a commercially available standard Forteo. The activity of the peptide was calculated by measuring the intracellular cAMP production after treating the UMR-106 cells with the peptides. The calculated specific activity of the prepared peptide was found to be $1.04X10^4$ IU/ mg protein.

Biological activity

The biological activity of the recombinant hormone was evaluated in a cAMP stimulation assay using the rat osteosarcoma cell line UMR-106. Chemically synthesized hPTH and Forteo were used as standards. Recombinant hPTH and the synthetic peptides showed similar dose response curves in the cAMP stimulation assay. The activity of the rhPTH(1-34) was estimated as $1.04X10^4$ IU/mg protein (Fig. 7). In vitro biological activity studies substantiated the biological activity of the recombinant hormone.

Conclusions

The method described here provides a rapid and efficient means for high-level expression and subsequent purification of PTH, and potentially a wide variety of other peptides, in E. coli. Numerous peptides of sizes comparable to that of hPTH(1-34) are increasingly becoming a focus

for biotechnology and pharmaceutical development.

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Pharmacokinetics and *In Vitro* and *In Vivo* Correlation of NN-dimethylaminocurcumin (NNDMAC) Loaded Polycaprolactone Microspheres in Rats

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Abstract

NN-dimethylaminocurcumin (NNDMAC), a novel curcumin analogue, has demonstrated significant hepatoprotective activity after oral administration. The objective of this investigation was to determine the pharmacokinetics of NNDMAC after the administration of its microsphere formulation. Additionally, it was aimed to determine the *in vitro in vivo* correlation (IVIVC) with the microsphere formulation. NNDMAC biodegradable microspheres were prepared using solvent evaporation technique by taking polycaprolactone as the polymer. A suitable release study based on the volume of distribution of NNDMAC was selected. *In vitro* release of the drug was determined. For *in vivo* studies, the microsphere formulation was injected by IP route. Pharmacokinetic properties of microsphere-encapsulated NNDMAC were determined and a comparison with i.v. solution form of NNDMAC was made. Pharmacokinetic analysis was performed using KINETICA and non-compartmental parameters were determined. Concentrations of the drug in plasma were determined by HPLC. IVIVC was established according to Drewe and Guitard (degree A). *In vivo* drug release into the systemic circulation was determined using Wagner-Nelson method. Results indicated that, when NNDMAC formulations were administered by IP route, mean residence time (MRT) and the area under the curve (AUC) were significantly higher ($P<0.05$) and maximum concentration (C_{max}) of NNDMAC was lower than that of the free form. T_{max} was same with both the administrations. The results obtained in the present study showed that microsphere encapsulated NNDMAC provides prolonged and effective plasma concentration after IP administration. The microsphere formulation sustained the release of the active for 9 days *in vitro* as well as *in vivo* in this rat model. Good IVIVC was achieved when the release medium selected was based on the volume of distribution of the drug.

Key words: NNDMAC, microspheres, sutained release, pharmacokinetics, IVIVC

Introduction

Curcumin and its analogues have been the subject of several pharmacological studies. Most of these studies were conducted with an intention to unravel their therapeutic potential and exploit the chemical structure for clinical use. Several bioactivities for curcumin and its synthetic analogues including their use in the cancers, tumors, alzheimers disease, inflammation, malaria, bacterial infections, neurological disorders, etc. have been reported (1). The analogues of curcumin were mainly

synthesized to increase the poor bioavailability of curcumin, its stability as well as solve the problems associated with its synthesis (2). Curcumin is of particular interest for a variety of pharmacological applications. Most studies involving its use do not obtain pure samples. Extractions from the natural product, turmeric, are the most common sources of curcumin. Purification is accomplished using extensive chromatographic extraction and is a very labor intensive endeavor that does not provide a very pure material suitable for pharmacological use. As an alternative to the extraction, synthesis of curcumin has been attempted. The synthesis of curcumin involves the use of relatively expensive components that require intensive removal of impurities that require treatment for their disposal (3). Other methods for the synthetic production of curcumin from the starting products vanillin and 2,4-pentanedione involve the use of tri-butyl borate, boron oxide, and butylamine in a hydrolysis reaction with N,N-dimethylacetamide as a solvent and recrystallization using acetonitrile. In this second approach, there are problems associated with the recovery, waste disposal and toxicity. Because of these problems with the synthesis of curcumin, synthesis of curcumin analogues was also accomplished as an alternative. Analogues of curcumin were synthesized using a variety of approaches (2). After synthesis, these compounds were screened for a variety of activities. Our group has synthesized several of 1,7-diaryl-1,6-heptadiene-3,5-diones, and in particular curcumin and its analogues. NNDMAC is one such analogue which possessed hepatoprotective, antidepressant and anti-inflammatory activities (4,5,6). Its chemical structure is shown in the Figure 1. We previously developed a biodegradable microsphere formulation, a parenteral depot system, for NNDMAC (6). The purpose of this study was to investigate the pharmacokinetics of NNDMAC microspheres in a rat model and also determine the IVIVC with the formulation. The data from this study adds knowledge to our quest to improvise therapy against several diseases where in curcumin analogues are useful. There are several advantages with biodegradable parenteral depot system (7). With these types of biodegradable parenteral depot systems, there is a possibility of patenting clinically successful drugs after incorporating them into newer drug delivery systems without infringing the original drug or formulation patents. Further, the development in the concepts and techniques of controlled release drug delivery systems coupled with the increasing expense bringing new drug entities to market, has encouraged the development of this new drug delivery system. It is also easy to deliver the novel, genetically engineered pharmaceuticals, i.e. peptides and proteins to their site of action without incurring significant immunogenicity or biological inactivation with this new drug delivery system. The basic rationale for controlled drug delivery is to alter the pharmacokinetics and pharmacodynamics of pharmacologically active moieties by using novel drug delivery systems or by modifying the molecular structure and physiological parameter inherent in selected route of administration. Thus, parenteral biodegradable depot microspheres is the attractive dosage form to be tested to enhance the pharmacokinetic properties as well the pharmacodynamic activity with selected curcumin analogues.

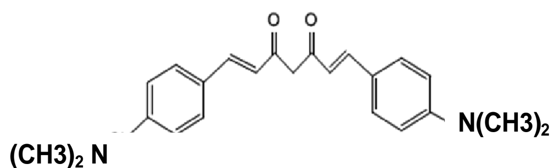

Fig. 1: Chemical Structure of NNDMAC

Materials and Methods

The required aromatic aldehyde p N N dimethylamino benzaldehyde was obtained from Merck. Benzene was purchased from Universal laboratories. Column silica gel was purchased from Finar chemicals limited. HPLC grade methanol and acetonitrile were purchased from Merk specialties. Methanol, ethyl acetate, and n-Hexane were purchased from Finar reagents. Acetone, benzene and toluene were purchased from Universal laboratories. Polycaprolactone was purchased from Sigma-aldrich, Germany. Ethanol LR, ethyl acetate, tween 80 and dichloromethane were purchased from Finar reagents. Benzene purchased from Universal laboratories was used. To conduct in vitro drug release studies, magnetic stirrer and cyclo mixer from Remi Equipments Pvt. Limited were used. A SL 164 Elico Double Beam UV-Vis Spectrophotometer was used to analyze the samples. HPLC from Cyberlabs was used for analysis of all the plasma and serum samples. Male Wister rats (100 to 150 gms, 5 to 6 weeks old) purchased from animal center of Mahaveera enterprises, Hyderabad were used in this study. A pharmacokinetic software KINETICA was used in the data analysis and the determination of pharmacokinetic parameters.

Synthesis and Characterization of NNDMAC

A mixture of acetyl acetone (0.01 mole), p N N dimethyl benzaldehyde (0.02 moles), boric acid (0.01 mole), in dimethyl formamide (10 ml), was taken into a round bottom flask (RBF) and few drops of diethanolamine and acetic acid mixture was added. The mixture was then refluxed in a mantel for 16 hours at 150^0C temperature. The reaction was monitored by TLC (Thin Layer Chromatography) for the confirmation of the product. After 16hrs of reflux the reaction mixture was poured into a 10% acetic acid solution and stirred for one hour to get a solid mass. Thus obtained mass was filtered and washed with water. This crude drug was purified and separated by column chromatography using 60-120 mesh TLC grade silica gel. The column was filled with silica gel of mesh size 60 to 120 and wet packing method was followed. The reaction product mixed with silical gel was loaded on top of the column and column was run with a mixture of n hexane and ethyl acetate (75:25) of 500 ml volume. The pure product was subsequently eluted by running the column with a mixture of methanol and benzene (50:50). The elutant was allowed to air dry. It was recrystallized by subsequent solubilization in benzene followed by methanol to get pure product. The purity of the compound obtained was confirmed using HPLC. A HPLC procedure employing a C-18, 100 X 4.6 column, SPD-10A UV-Vis detector, LC-10 AD pump and C-R7A Plus integrator was used. HPLC grade methanol and water in the ratio of 70:30 was taken as the mobile phase. The detection wavelength was 425 nm. Further, the structure was confirmed using NMR.

Fabrication of NNDMAC Microspheres

Microspheres of NNDMAC using biodegradable polycaprolactone as the polymer were fabricated using emulsion-solvent evaporation method. Dichloromethane was taken as organic phase in which polymer (400mg) and drug (200mg) in a ratio of 2:1 were dissolved (20ml). This organic phase was added to the aqueous phase containing tween 80 as surface active agent (1% w/v solution) drop by drop while the aqueous phase was kept for stirring on a magnetic stirrer. Stirring was continued till complete evaporation of dichloromethane occurred. As the organic phase evaporates precipitation of the polymer and drug occurs due to which drug gets entrapped in the polymer and

stirring results in size reduction as well as spherical particle formation.

In vitro Drug Release Studies

A dialysis membrane was used for the release study. The release medium, PBS (7 ml) was taken into the receiver compartment. Release medium was designed based on the volume of distribution. The volume of distribution of this drug in rats was ~ 7 ml (5). This has been selected so as to obtain good *in vitro – in vivo* correlation. The donor compartment was immersed into the receiver compartment so that the edge just touches the receiver compartment. A 100mg of the microparticles were dispersed in 2 ml of PBS and placed in the donor compartment and of this suspension 1 ml was used in the release studies. The percentage loading of the drug was found to 70% and as a reason, the 50 mg of microsphere suspension used in the release studies contained 35 mg of the drug. The rpm of the system was maintained using magnetic stirrer and bead. Samples (1 ml) were removed from the receptor compartment and replaced with fresh medium immediately. The samples were then analyzed for the drug. A n=3 was used in the study and the data is reported as mean ± S.D. The release studies were also conducted with the pure drug so as to show the sustained release of the drug from the microspheres. This set of release studies were exactly similar to that conducted using the microspheres, excepting the use of pure drug in this case. The amount of the drug taken in the release studies was also the same (35 mg). Before selecting the wavelength to be used in the analysis of the compound, a UV spectrum of the compound in PBS and methanol was generated. The UV spectrum of the compound in the release medium was also generated. Based on the results and the sensitivity, the samples were analyzed using a UV-visible spectrophotometer at 425 nm wavelength.

Pharmacokinetic Study

The study was conducted in rats after getting approval from ethical committee constituted for this project in Vaagdevi College of Pharmacy, Warangal, AP, India. Male wistar rats (250 g) were purchased from Mahaveer Enterprises, Hyderabad. Animals were maintained in an air-conditioned room at 22 ± 2º C and relative humidity of 45-55% under a 12 h light:12 h dark cycle. The animals had free access to standard food pellets and water was available ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Vaagdevi College of Pharmacy, Warangal (Registration No: 1047/ac/07/CPCSEA) and constituted in accordance with the rules and guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPSEA), India. After quarantine period, rats were divided into two groups (n=4). One group was administered drug solution via i.v. route while other group was injected with biodegradable microspheres intraperitoneally. A 35 mg of the drug dissolved in sterile PEG 400 was used in the i.v. solution administration. A 400 mg of the NNDMAC microspheres were dispersed in 4 ml of normal saline and a 500 µl of this suspension containing 35 mg of the drug were injected into each rat. Blood samples (0.5 ml) were collected from retro orbital sinus of rat eye under anesthesia at intervals of 0.25, 0.5, 0.75, 1, 3, 6, 12, and 24 hrs in case of i.v. solution. For microparticular system along with above time intervals samples were also collected after 4 and 9 days. The blood samples so collected were added to a series of graduated micro centrifuge tubes containing 0.3 ml of sodium citrate solution (4% w/v in water). All the samples were centrifuged at 3000 rpm for 10 minutes and plasma was separated into other micro centrifuge tube by using micro pipette and stored in deep freeze. The drug was extracted

from the plasma by adding 500 µl of ethyl acetate, and vortexed on cyclo mixer for 20 min. The organic phase was separated and collected into another micro centrifuge tube and allowed to air dry by keeping the lid of the tube open for 24 hours. These dried tubes were stored in deep freeze until HPLC analysis was performed. HPLC analysis samples were reconstituted with 50 µl of mobile phase (methanol: water, 70:30) and analyzed at 230nm wavelength. The wavelength was selected based on the sensitivity and specificity of the compound in a HPLC UV-Vis detection. The HPLC method was validated for inter day and intraday variability's. From the plasma data various pharmacokinetic parameters were determined.

In Vitro In Vivo Corelation

There are four levels of IVIVC that have been described in the FDA guidance, which include levels A, B, C, and multiple C (8). Here the correlation was established according to Drewe and Guitard basing on degree A. The parameters compared were cumulative absorption profile to that of in vitro dissolution profile i.e. correlation of the amount of drug dissolved to that of respective fraction of dose absorbed (T_{50}). Cumulative amount of the drug absorbed was calculated using Wagner-Nelson method approximating the kinetics of the drug to one compartment open model. According to Wagner-Nelson method, the cumulative amount of drug released from the microspheres into the systemic circulation in a rat was calculated as given below:

$$A_b / A_b^{\infty} = (C_p + K[AUC]_0^t) / K[AUC]_0^{\infty}$$

Where A_b is the cumulative amount released at any time, A_b^{∞} is the dose administered, C_p is the plasma concentration at any time t, K is the elimination rate constant and AUC is the area under the curve. K was also determined in this study in another set of rats where NNDMAC was administered via i.v. route. To determine the IVIVC with the formulation, the percentage of NNDMAC dissolved from microspheres was plotted on X-axis and the corresponding NNDMAC absorbed in the rats from the microspheres was plotted on the Y-axis and this graph was fitted to a straight line and the correlation coefficient was determined. This correlation indicates the strength of IVIVC of this study.

Data Analysis

All data in this study were presented as mean or mean±SEM. Data were analyzed by t-test. Significance was recognized at $P < 0.05$.

Results

The drug was synthesized successfully using protocol followed in this study. After synthesis, the structure of NNDMAC was confirmed using NMR. The NMR results are as follows: 7.6-7.8(m, 8H, Ar); 6.1(d, 2H, HC=CH); 4.9(d, 2H, HC=CH); 3.3(s, 2H, CH_2); 2.2(s, 12H, $N(CH_3)_2$). A UV-Vis spectrum was generated so as to identify the wavelength to be used in the assay of the compounds. The spectrum indicated two different λ_{max} values which were 230 nm and 425 nm. The drug assay at 425 nm was used to analyze the in vitro release samples while the assay at 230 nm was used in the HPLC. This is because the sensitivity of the assay was higher at 230 nm in a UV-Vis detector used in the HPLC and the specificity of the assay was higher at 425 nm when a UV-Vis spectrophotometer was used. At 230 nm, there was a significant interference from the degradation products of the polymers in the in vitro release studies with

that of the drug and this interference was not noted at 425 nm, suggesting more specificity of the assay at 425 nm in a UV-Vis spectrophotometer. Thus, this wavelength was also used in the assay of the drug after synthesis. In this case, the starting material used in the synthesis also demonstrated a λ_{max} at 230 nm. The assay at 230 nm in HPLC for plasma samples was used because of the higher sensitivity when compared to that at 425 nm. The HPLC method used was validated for interday and intraday variabilitys. The results of intra and inter-day variation of NNDMAC at three different concentrations levels (Level 1, Level 2 and Level 3) were determined**.** The data indicates that the maximum %relative error at Level 1, Level 2 and Level were 1.8, 1.72 and -1.99, respectively while the maximum % relative standard deviation was 1.5, 1.9 and 1.4 respectively indicating that the method has acceptable accuracy and precision. Also, the calculated t-values were lesser than the tabulated t-value of 4.3 for $\alpha = 0.05$ at two degrees of freedom. This indicated that the experimental values were not significantly different from the nominal which reflected the accuracy of the method. Further, one way ANOVA was performed to get estimates of within and between day variability. The calculated F value was lesser than the tabulated $F_{2,6}$ ($\alpha = 0.01$) of 10.92 indicating that the inter day variability was not significantly different from the intra day variability at 1% level of significance**.**

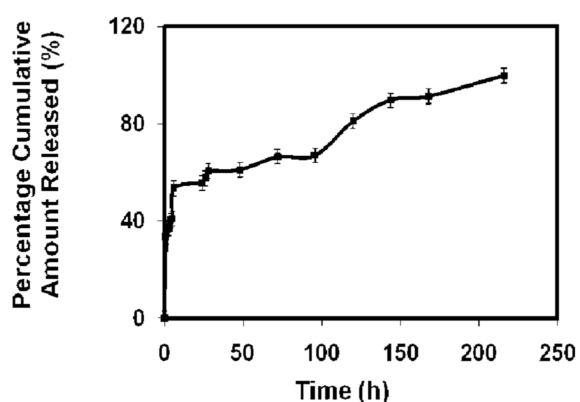

Fig. 2: *In Vitro* Drug Release from NNDMAC Microspheres

The drug release *in vitro* from the microspheres was sustained for 9 days (Figure 2). A 99.8% drug was released during this time. The rest of the drug could be the drug irreversibly bound to the polymer. The release of the drug from pure samples was also investigated. A 100% of the entire pure drug taken at similar quantities was released within 48 hours under similar conditions. This confirms the sustained release of the drug from the microsphere formulations. Plasma concentration vs time curve after single IV bolus solution and IP formulation (microsphere) administration are shown in the Figure 3. Drug concentration started to be detected in the plasma from 1 hour onwards. Drug release was sustained *in vivo* after the microsphere administration. After IP administration of the microspheres, the noncompartmental parameters, area under the concentration time curve (AUC), MRT, t_{max}, and C_{max} were determined using KINETICA. All the noncompartmental PK parameters for the i.v. bolus administration and microsphere administration are shown in Table 1. After administration NNDMAC microsphere formulation via the IP route, mean residence time (MRT) and the area under the curve (AUC) were significantly higher ($P<0.05$) and maximum concentration (C_{max}) of NNDMAC was lower than that of the free form. T_{max} for both the administrations was the same. To determine degree A IVIVC, the amount of the drug absorbed was determined using Wagner-Nelson. The cumulative percentage of drug dissolved and

PK Parameter	IV Solution Administration	IP Microsphere Administration
Cmax (µg/ml)	2.4 ± 0.2	3.2±0.3
Tmax (h)	1 ±0.2	1 ±0.1
AUC (µg*h/µl)	0.025 ±0.01	0.055 ±0.005
MRT (h)	9.3 ±2	131.47 ±3
R^2	0.997	0.954

Table 1 Pharmacokinetic Variables of NNDMAC after single IP and IV Administration of Microspheres and Solution (Mean±SEM)

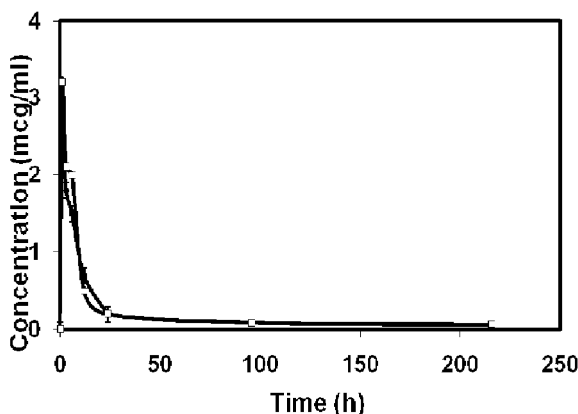

Fig. 3: Plasma Log(Conc) vs Time Profile of NNDMAC After IV Administration of Solution (—-◆——) and IP Administration of the Microsphere Formulation (——□——-)

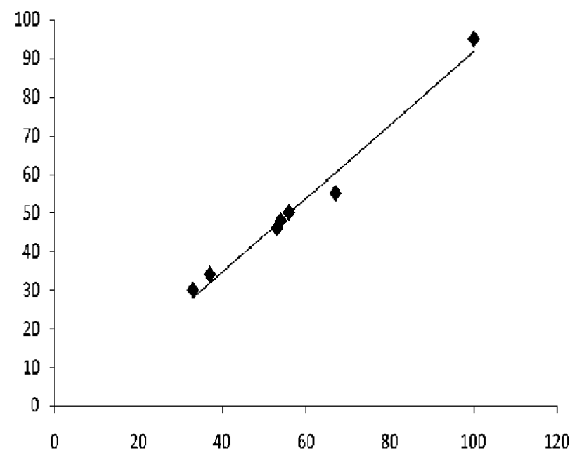

Fig. 4: In vitro In Vivo Correlation of Drug Release From the Microspheres

cumulative fraction of drug absorbed were compared. The graphical analysis confirms a good degree of correlation ($r^2 = 0.982$) (Figure 4). Thus, it can be concluded that good IVIVC was obtained when the volume of distribution was used as the volume of the release study.

Discussion

Optimal design of controlled release systems requires a through understanding of pharmacokinetics of drug with and without the delivery system (9). Also the comprehension of in vitro in vivo correlation (IVIVC) of the delivery is essential to better tailor the delivery system for the future needs (8,10). The development of new injectable drug delivery systems has received considerable attention over the past few years. This interest has been sparked by the advantages this delivery system possess, which include ease of application, localized delivery for a site specific

action, prolonged delivery periods, decreased body drug dosage with concurrent reduction in possible undesirable side effect common to most forms of systemic delivery and improved patient compliance and comfort. Thus, we developed NNDMAC microspheres and in this study, investigated the pharmacokinetics and IVIVC of the active. The developed formulation can be used where in the curcumin analogues can be used for therapeutic purposes. The pharmacology of curcumin and its analogs is complex, with extensive metabolic conversions involved in the activation, inactivation and elimination of the drug. It is cleared via glucuronidation. These drug properties also contribute to the marked heterogeneities in efficacy observed with curcumin and its analogs. Hence, drug carrier technologies represent a rational strategy to improve pharmacokinetics thereby enhancing the pharmacodynamics of the drug. After IP administration of the microspheres, drug concentration reached C_{max} within a longer time ($P<0.05$) than that of solution forms. The results are similar to studies performed with other drugs, administration routes and species (11). The results suggest that the absorption of NNDMAC after microsphere administration from injection sites was slower. In this study, it was determined that the C_{max} of NNDMAC with microsphere administration was lower than that obtained out of injecting solution. After IP administration of microsphere encapsulated drugs, which acted as a local depot, there was a slower release, lower C_{max}, and long-lasting concentrations of active agent in the plasma compared with administration of the free form. After IP administration of microsphere formulation, the low C_{max} and plasma concentrations may cause a reduction in dose-dependent side effects of the drug (12). In the present study, MRT of NNDMAC from microspheres was longer than those of the drug obtained out of injecting a solution. These results suggest that microsphere encapsulated drug formulations provide longer effective concentrations in plasma. The microspheres of this study were prepared using a biodegradable polymer polycaprolactone. Polycaprolactone (PCL) is a biodegradable polyester with a low melting point of around 60°C and a glass transition temperature of about "60°C (13). PCL is degraded by hydrolysis of its ester linkages in physiological conditions (such as in the human body) and has therefore received a great deal of attention for use as an implantable biomaterial. In particular it is especially interesting for the preparation of long term implantable devices, owing to its degradation which is even slower than that of polylactide. PCL is an Food and Drug Administration (FDA) approved material that can be used in the human body as (for example) a drug delivery device. A variety of drugs have been encapsulated within PCL beads for controlled release and targeted drug delivery.

The second objective of this study was to evaluate the IVIVC with the microsphere formulation. A predictive IVIVC can empower in vitro dissolution as a surrogate for *in vivo* bioavailability/biotheraequilance (8,10). IVIVCs can decrease regulatory burden by decreasing the number of biostudies required in support of a drug product. Additionally, IVIVC is also helpful in the product development including the development of depot microspheres. The development of an IVIVC is a dynamic process starting from the very early stages of development program through the final step. Different types of IVIVCs are used in the regulatory terminology. These include assumed IVIVC, retrospective IVIVC, and prospective IVIVC. An assumed IVIVC is essentially one that provides the initial guidance and direction for the early formulation development activity. Thus, during stage 1 and with a particular product concept in mind,

appropriate *in vitro* targets are established to meet the desired *in vivo* profile specification. This assumed model can be the subject of revision as prototype formulations are developed and characterized *in vivo*, with the results often leading to a further cycle of prototype formulation and in vivo characterization. Out of this cycle and *in vivo* characterization and, of course, extensive *in vitro* testing is often developed what can be referred to as retrospective IVIVC. With a defined formulation that meets the *in vivo* specification, Stage 2 commences. At this stage based on a greater understanding and appreciation of defined formulation and its characteristics, a prospective IVIVC is established through a well defined prospective IVIVC study. Once the IVIVC is established and defined it can be then used to guide the final cycle of formulation and process optimization leading into Stage 3 activities of scale-up, pivotal batch manufacture, and process validation culminating in registration, approval and subsequent post-approval scale-up and other changes. In this study, a part of retrospective IVIVC was accomplished based on the volume of the distribution to be the volume used in the release studies. It is taken under the assumption of one definition of volume of distribution and the definition is "It is the hypothetic volume of the body compartment in which the drug is distributed". Although this definition is still controversial it would most likely benefit our study in establishing the retrospective IVIVC we aimed at. Thus, the release was performed in 7 ml of PBS based on the volume of distribution (5). The aim of such a study is to obtain good *in vitro – in vivo* correlation. Ideally, physiological conditions at the site of administration should be taken into account when selecting the *in vitro* dissolution/release test conditions. The complexity of the release mechanism of some novel/special dosage forms and the lack of knowledge about the conditions under which release occurs *in vivo* make it difficult to design physiologically based tests in all cases, but it should be possible to conceive a test that can detect the influence of critical manufacturing variables, differentiate between degrees of product performance, and to some extent characterize the biopharmaceutical quality of the dosage form. As the release mechanism and site of application vary dramatically among the novel/special dosage forms, the experimental test conditions have to be tailored according to the conditions at the site of administration (eg, temperature of the test) and the release mechanism (eg, chewing gums will require different agitation rates than suspensions). Within a given category, it may be necessary to have product type-specific dissolution tests (eg, separate tests for lipophilic and hydrophilic suppositories), and in some cases for products containing the same drug and administered in the same type of novel/special dosage form but with a different release mechanism (analogous to the range of tests available in the USP for theophylline extended release dosage forms). Several studies also used volume of distribution to be volume to be used in the release medium especially for sustained release dosage forms. In this study also, we used similar approach.

The correlation was established according to Drewe and Guitard basing on (degree A) i.e., the comparison of cumulative absorption profile and cumulative *in vitro* release profile was made. A level A correlation of in vitro release and in vivo absorption could be obtained for individual plasma level data by means of the Wagner and Nelson method. This type of evaluation of in vivo absorption was previously applied for drug delivery systems. To develop level A correlation the estimation of the *in vivo* absorption or dissolution time course is performed using an appropriate deconvolution techniques such as Wagner-Nelson procedure or Loo-Riegelman

method or numerical deconvolution for each formulation and subject. Wagner-Nelson and Loo-Riegelman methods are both model dependent in which the former is used for a one-compartment model and the latter is for multi-compartment system. However, Wagner-Nelson method is less complicated than the Loo-Riegelman as there is no requirement for intravenous data. However, misinterpretation on the terminal phase of the plasma profile may be possible in the occurrence of a flip-flop phenomenon in which the rate of absorption is slower than the rate of elimination. To avoid complicated calculations which are bound by regression parameters and make the analysis simpler, we used Wagner-Nelson method in this study. Further, the presence or absence of flip-flop in the pharmacokinetic data was also verified using i.v. bolus data. Additionally, these techniques represent a major advance over the single-point approach in that these methodologies utilize all of the dissolution and plasma level data available to develop the correlations. Good IVIVC was observed in this retrospective IVIVC study. The correlation made according to the volume of distribution is suitable for NNMDAC microsphere. The same methodology should be investigated for other drugs so as to further add a speck of knowledge to this type of establishment of IVIVC.

Conclusion

In conclusion, when microsphere formulations are compared to solution, formulation demonstrated a lower Cmax, higher MRT and provides effective and prolonged plasma concentration in the body after IP administration. In addition, when NNDMAC was administered in a microsphere entrapment form it had long duration of activity threshold, this result may be beneficial for the diseases in which NNDMAC is useful. Good IVIVC was obtained when the volume of distribution was used as the volume of the release study.

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Evaluation of *in-vitro* Cultured Cells of *Withania somnifera* for Antioxidant Activity

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Abstract

Withania somnifera (Solanaceae), commonly known as ashwagandha, is known to have anti-inflammatory, antitumor, anticonvulsive and immunosuppressive properties. In the present study, antioxidant potential of *in vitro* cultured cells and roots of *W. somnifera* was evaluated within the concentration range of 5-100 µg/ml using *in vitro* studies *viz.* free radical scavenging capacity on 1, 1-diphenyl,- 2-picrylhydrazyl (DPPH) radical, 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) radical cation decolourization assay, scavenging of nitric oxide radical and total antioxidant capacity. Ascorbic acid was used as standard compound. Among antioxidant screening models tested, ethanolic extracts of *W. somnifera* cells from transformed callus cultures had shown better antioxidant potential in comparison to that of roots and ascorbic acid. Therefore the results justify the therapeutic application of *W. somnifera* as an antioxidant in the indigenous system of medicine.

Key words: Antioxidant, Free radical scavenging capacity, *Withania somnifera*, Withanolides.

Introduction

Generation of free radicals is known to be involved in the development of various ailments like cancer, diabetes, liver cirrhosis, nephrotoxicity, Alzheimer's disease, parkinsonism and inflammatory responses (1-4). These free radicals are produced in body as by products of biological redox reactions (5) and their concentrations exist in a dynamic equilibrium with antioxidants to quench and/or scavenge and then to protect the body against harmful effects of free radicals. Reactive oxygen species (ROS) mainly causes cumulative damage of DNA, proteins, lipids and membranes and thus oxidative stress. This may result in chromosomal aberrations and/or genetic alterations leading to carcinogenesis (6). Furthermore, imbalance between pro-oxidant and antioxidant homoeostasis results in various degenerative diseases. Presently, the use of complimentary and alternative therapy and especially the utilization of phytoconstituents have been significantly increasing worldwide. This is due to better acceptance of herbal products than synthetic drugs because of lesser side effects and better compatibility thus improving patient tolerance even on long-term use (7). Crude extracts from plants and a number of phytochemicals are known to posses excellent antioxidant potential and may serve as successful lead molecules in therapeutic armamentarium against these diseases.

Withania somnifera, also known as ashwagandha or Indian ginseng has been an important herb in the Ayurvedic and indigenous medical systems for more than 3,000 years (8). It has received much attention in recent years due to the presence of a large number of steroidal alkaloids and lactones known as withanolides.

At present, 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. The principle withanolide in the Indian variety of the plant is withaferin A. This drug is known to have anti-inflammatory (9), antitumor (10), anticonvulsive (11) and immuno-suppressive properties (12). Preparation of crude extract from natural sources by solvent extraction method generally resulted in low content of therapeutically active phytoconstituents due to presence of impurity. Therefore crude extract prepared from *in vitro* cultured cells may provide a suitable alternative. Keeping this in view, callus cultures of *W. somnifera* was established in the present study. Crude extract from *in vitro* cultured cells of *W. somnifera* was evaluated for its antioxidant potential and compared to that of roots.

Materials and methods

Collection of plant material and germination of seeds

Plant materials (seeds and roots) of *W. somnifera* were collected from Neemuch district of Madhya Pradesh. The dried roots of the plant were used for extraction. Seeds were used to develop *in vitro* plants for initiation of cell cultures. Seeds of *W. somnifera* were washed in 1% savlon and then treated with 0.1% bavestin and rinsed five to six times with sterile double-distilled water (SDDW). Surface sterilization was performed using 70% v/v ethanol treatment for 30 s and rinsed thrice with SDDW. This was followed by treatment with 0.01% w/v mercuric chloride for 5 min and rinsing with SDDW for four to five times. For aseptic germination, sterilized seeds were then placed on Murashige and Skoog (MS) medium (13) with 30 g/l sucrose and 7 g/l agar at 25±2° C in a 16/8-h light/dark cycle with a light intensity of 1,200 lux.

Initiation of transformed callus cultures

Hypocotyls were used as explants for transformation by *Agrobacterium tumefaciens* strain (MTCC 2250) procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. . Explants from 25-day-old *in vitro* germinated plants were used for culture initiation studies. For this, bacterial colonies were cultured for 2 days on solid yeast mannitol broth medium (YMB) at 25±2° C. The culture (2% v/v) was reinoculated in liquid YMB medium and grown till they achieved an optical density of ~ 1.0 at 600 nm. The suspension was then centrifuged at 6,000 g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 5 ml of fresh liquid YMB media. This concentrated culture was used further for the infection of plant materials.

Forty explants were kept in a sterile plate, pricked manually with a 24-gauge metal needle (~5 wound per cm^2), dipped in *Agrobacterium* culture, and incubated for 5 min. The liquid YMB medium without bacteria was applied to the explants as a control. The infected explants were preincubated for cocultivation at 25±2° C for 48 h on sterile MS medium, solidified with 10 g/l agar. The infected explants were then transferred to an antibiotic, cefotaxime (1 g/l) containing MS medium to check the overgrowth of bacteria and were incubated at 25±2° C in 16/8-h light/dark regime. The transformed cultures were then transferred to fresh MS medium containing 1 g/l cefotaxime. Axenic cultures were obtained by subsequent subculture to fresh MS medium for every 7 days containing the antibiotic. Transformed cultures were checked for *Agrobacterium* contamination by culturing samples on YMB after every subculture (14). Axenic cultures of *W. somnifera* were maintained on 100 ml of MS medium solidified with 8 g/l agar in 500 ml Erlenmeyer flasks by transferring 2 g

of fresh water to each flask. Cells were harvested after 20 days for extraction.

Preparation of extracts and phytochemical tests

The collected roots and *in vitro* cultured cells of *W. somnifera* were air dried at 27±2° C, powdered and stored in an air tight container at 27±2° C till further use. These dried and powdered roots and cells (200 g each) were accurately weighed and defatted with 1 litre petroleum ether (40-60). It was then extracted separately with 1 litre ethanol in a soxhlet for 36 h. The extracts were filtered, evaporated to dryness under vacuum and stored in the desiccators for use in subsequent experiments. The qualitative chemical investigations of ethanolic extracts obtained from *in vitro* cultured cells and air dried roots of *W. somnifera* were carried out to check the presence of various phytoconstituents (15).

Assessment of anti-oxidant activity

The assessment of anti-oxidant activity was carried out using following methods:

1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

For the present study, the samples were prepared in different concentrations *i.e.* 5-100 µg/ml in methanol. The samples of above concentrations were mixed with 3 ml of 100 µM DPPH prepared in methanol and final volume was made up to 4 ml with methanol. The absorbance of the resulting solutions, in triplicate, and the blank (with same chemicals except sample, if required) were recorded after 20 min at 25±2° C against ascorbic acid. The disappearance of color was read spectrophotometrically at 517 nm using a Shimadzu visible spectrophotometer. Radical Scavenging Capacity (RSC) in percent was calculated by following equation:

$$RSC (\%) = 100 \times [A_{blank} - A_{sample} / A_{blank}]$$

Where;

RSC = Radical Scavenging Capacity,

A_{blank} = Absorbance of blank,

A_{sample} = Absorbance of sample,

From the obtained RSC values, the IC_{50} were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization (16).

ABTS radical cation decolorization assay

ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS solution (7 mM) with 2.45 mM ammonium persulfate and the mixture was allowed to stand in dark at 25±2° C for 12-16 h before use. For this study, different concentrations (5-100 µg/ml) of the ethanolic extracts (2 ml) were added to 1.2 ml of ABTS solution and the final volume was made up with ethanol to 4 ml. The absorbance was read at 745 nm and the experiments were performed in triplicate (17).

Scavenging of nitric oxide radical

Nitric oxide is generated from sodium nitroprusside and measured by Griess' reaction (18,19). Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M, pH: 7.4) was incubated with different concentrations (5 -100 µg/ml) of the ethanolic extracts dissolved in phosphate buffer saline and the tubes were incubated at 25±2° C for 5 h. Control experiments without the test compounds but with equivalent amounts of buffer were conducted in an identical manner. After 5 h, 0.5 ml of incubation solution was removed and diluted with 0.5 ml of Griess reagent (1 % sulphanilamide, 2 % *O*-phosphoric acid and 0.1 % naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylenediamine was recorded at 546 nm. The experiment was repeated in triplicate (20).

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Total antioxidant capacity

Ethanolic extracts of *W. somnifera* (100 µg) were added, in triplicate, to a mixture of ammonium molybdate (4 mM) and sodium phosphate (28 mM) in 0.6 M H_2SO_4 in total volume of 2 ml in eppendorff tubes and kept at 95 ±2° C for 90 min and the absorbance was measured at 695 nm after cooling at 25±2° C (21).

IC_{50} was calculated by using formula:

$$b = \Sigma x.y/\Sigma x^2$$

$$a = y - bx$$

$$IC_{50} = a+b\,(50)$$

where, b = Regression coefficient of x on y ; a = Intercept of the line ; x = Concentration in µg/ml; y = % Scavenging; x = Mean of concentration; and y = Mean of % scavenging.

Statistical analysis

The results were expressed as mean values. The significance of statistical analysis was performed by ANOVA followed by Dunnett's test and P values (< 0.05 and < 0.01) implied significance.

Results and Discussion

In present study, transformed callus cultures of *W. somnifera*, capable to produce withaferin A, were developed and evaluated for antioxidant potential. DPPH radical scavenging capacity, nitric oxide scavenging effect and ABTS assay were used for establishment of antioxidant potential of *W. somnifera* in comparison to ascorbic acid as standard compound within the concentration range of 5-100 µg/ml. Extraction of roots and cells of *W. somnifera* using soxhlet apparatus resulted in crude extracts with yields of 13.4 % w/w and 12.3 % w/w respectively. Preliminary phytochemical screening of ethanolic extracts revealed the presence of steroids and alkaloids. Their presence was further confirmed qualitatively by thin layer chromatographic studies. DPPH is a relatively stable free radical and this method determines the ability of ethanolic extract of *W. somnifera* roots (TER) and *in vitro* cultured cells (TEC) to reduce the DPPH radical to the corresponding hydrazine by reacting with the hydrogen donors in the antioxidant principles (22). DPPH radicals convert the unpaired electrons to the paired one and the solution loses colour stoichiometrically depending on the number of electrons taken up (23). The dose dependant inhibition of DPPH by extracts (TEC and TER) and ascorbic acid is given as fig. 1. IC_{50} values of 32.55 µg/ml, 40.4 µg/ml and 52.5 µg/ml were obtained from ethanolic extracts from cells from callus cultures (TEC), roots (TER) and ascorbic acid respectively. Ethanolic extracts of *W. somnifera* exhibited better antioxidant potential in comparison to ascorbic acid as evidenced by lower IC_{50} values respectively in DPPH assay.

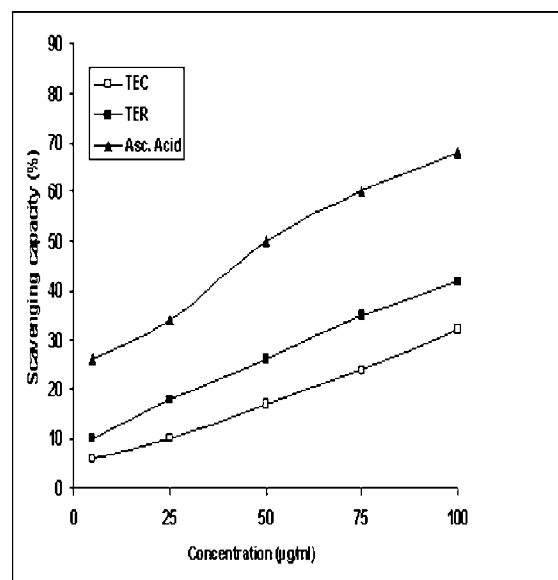

Fig. 1: DPPH scavenging capacity of *W. somnifera* extracts and ascorbic acid [TEC - Total extract from *in-vitro* culture cells, TER - Total extract from roots, Asc. acid - Ascorbic acid]

The ABTS decolourization assay results in direct generation of ABTS radical mono cation prior to addition of antioxidant components instead of in presence of it. The concentration dependent inhibition/scavenging properties of TER, TEC and ascorbic acid towards $ABTS^{\cdot+}$ are given as fig. 2. TEC had exhibited comparatively higher antioxidant potential with IC_{50} value of 41.8 µg/ml in comparison to TER and ascorbic acid with respective IC_{50} values of 48.9 µg/ml and 51.56 µg/ml.

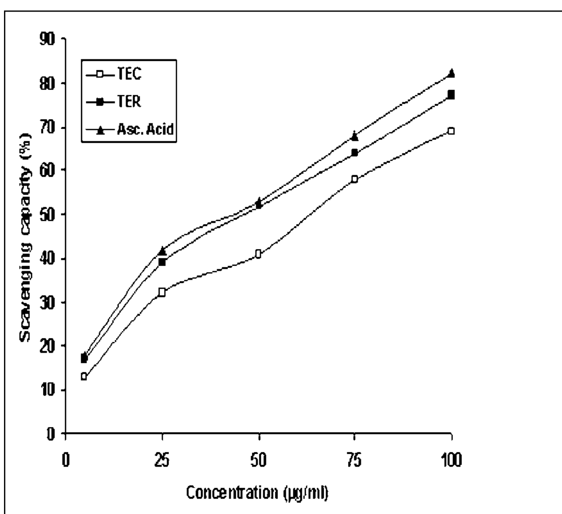

Fig. 2: ABTS scavenging capacity of *W. somnifera* extracts and ascorbic acid [TEC - Total extract from *in-vitro* culture cells, TER - Total extract from roots, Asc. acid - Ascorbic acid]

Chemically NO is very unstable under aerobic condition and it produces nitrite and nitrate upon reacting with O_2 through intermediates like NO_2, N_2O_4 and N_3O_4. In the present study, incubation of sodium nitroprusside in phosphate saline buffer had resulted in generation of nitrite, which was reduced by ethanolic extracts of *W. somnifera*. This effect may be due to competition of antioxidant compounds present in extract with oxygen to react with nitric oxide (24), which ultimately leads to inhibition of generation of nitrite. Effect of different concentrations of ethanolic extracts (TER and TEC) on nitric oxide scavenging capacity was determined and results are presented as fig. 3. The extracts, TEC and TER, exhibited scavenging potential with IC_{50} values of 17.55 µg/ml and 25.69 µg/ml respectively. These values were significantly lower than ascorbic acid (46.67 µg/ml) used as standard in the assay indicating higher antioxidant activity of extracts of *W. somnifera*. Total antioxidant potential of extracts was also determined by formation of phosphomolybdenum complex under acidic conditions. Ethanolic extract of *in vitro* cultured cells exhibited slightly higher total antioxidant activity (326) in comparison to that of roots of *W. somnifera* (264).

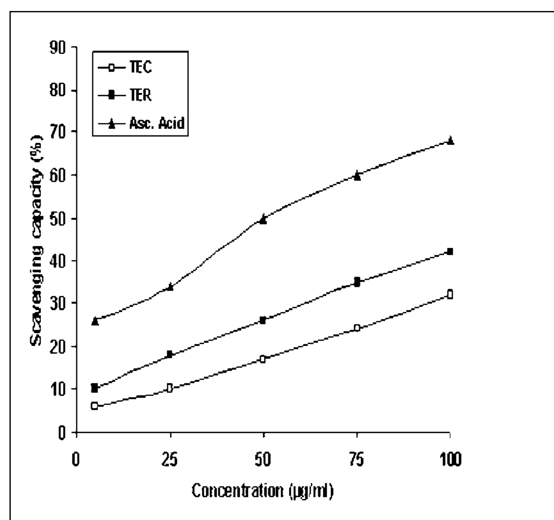

Fig. 3: NO scavenging capacity of *W. somnifera* extracts and ascorbic acid [TEC - Total extract from *in-vitro* culture cells, TER - Total extract from roots, Asc. acid - Ascorbic acid]

Conclusion

The present study proved promising anti-oxidant potentials of ethanolic extracts of *W.*

somnifera. It is reported that secondary products have good antioxidant activities. Therefore the antioxidant activity of *W. somnifera* may be attributed to the presence of these compounds. Furthermore ethanolic extract of *in vitro* cultured cells had shown better free radical scavenging capacity in comparison to roots. This might be due to presence of other compounds/impurities in ethanolic extracts from roots. Hence these results support the view that some traditionally used Indian medicinal plants are a promising source of potential antioxidants.

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Gibberellic Acid and Cycloheximide Influenced the Growth and Biochemical Constituents of a Medicinally Important Plant - *Trachyspermum ammi* (l.) Sprague

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Abstract

The effect of growth promoter gibberellic Acid (GA_3) and growth inhibitor cycloheximide (CH) was tested on seedlings of a medicinally important plant - *Trachyspermum ammi.* The seedlings under the GA_3 influence showed enhanced germination, seedling elongation and dry weight accumulation, and retarded effect on moisture absorption. The CH had inhibitory effect on percentage of germination, moisture content and seedling elongation, while dry weight accumulation was more. Both GA_3 and CH showed stimulatory effect on reducing sugars with decline in total sugar levels, whereas protein content enhanced with retarding levels in total amino acids.

Keywords: Caraway, GA_3, Growth, Cycloheximide, Reducing sugars, Total sugars, Total amino acids, Total Proteins.

Abbreviations: GA_3: Gibberellic Acid; CH-Cycloheximide;

Introduction

Trachyspermum ammi (Ajowan caraway, family Apiaceae) is an erect, minutely pubescent, branched annual, medicinally important plant cultivated in many parts of India, Pakistan, Afghanistan, Egypt and Europe (1). *T. ammi* is widely cultivated for its essential oil in which phenols, thymol and carvacrol are important constituents. The Indian pharmacopoeia requires Ajowan oil to contain not less than 40 percent thymol. Preliminary studies of oil indicated that it is a hypotensive agent and seems to possess anti-diuretic effect. *T. ammi is* known for its antiviral (2), anti-inflammatory (3), antifungal (4-8), molluscicidal (9-11), antihelminthic (in sheep) (12), plant nematicidal (11), antipyretic (13), antiaggregatory (14) and antimicrobial activity (15-17). *T. ammi* possesses *in vivo* antifilarial activity against the human filarial worm *B. malayi* in *Mastomys coucha*, macrofilaricidal activity and female worm sterility *in vivo* against *B. malayi* (18), besides inhibition of platelet aggregation (19), antifungal effects (20) and decrease of blood pressure (21). Very recently *T. ammi* seed powder was shown to have antihyperlipedemic effect with decrease in LDL-cholesterol, an increase in HDL-cholesterol and a decrease in plasma triglycerides (22). Considering the importance of the species, germination problems in *T. ammi* have been attempted to study the effect of growth regulators on seed germination and seedling growth, with special emphasis on total content of amino acids, proteins and sugars.

Materials and Methods

Seeds of *T. ammi* were obtained from Spice Research Station, Gujarat Agricultural University, Jagudan, Gujarat, India and were washed under

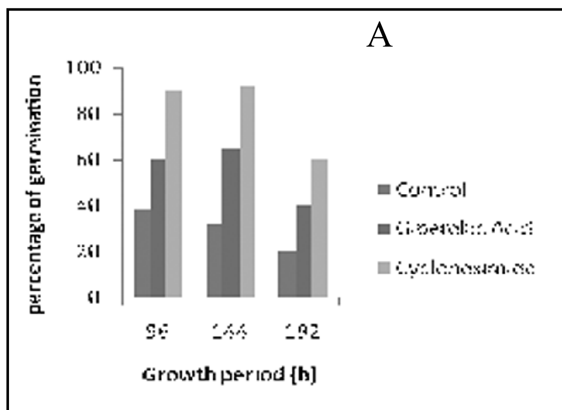
running tap water for 30 min and were surface sterilized with a mixture 10% NaOCl and 2% SDS (10% w/v) for 3 min. The surface sterilized seeds were washed thrice with sterile distilled water and dried on sterilized tissue paper. Seedlings were developed in the Petri dishes supplementing the respective growth regulators gibberellic acid (GA_3) (10 ppm) and cycloheximide (CH) (15 ppm) and distilled water as control. The renewal of solution and filter papers was carried out at 48 h interval. All the cultures were incubated at 25^0C under 16/8 h (dark/light) photoperiod with light intensity 40 -50 μ mol $m^{-2}s^{-1}$ provided by cool white fluorescent lights. The number of seeds germinated under GA_3 and CH was noted at an interval of 48 h until maximum germination attained. Germinability is expressed as percentage of germination.

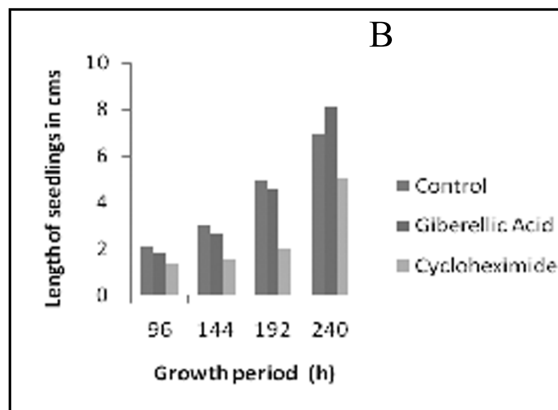
The exact fresh weight of the seedlings selected randomly from different treatments was taken. To record the dry weight, the seedlings were weighed individually and kept in an oven at 100 – 105 ^{0}C for three days or until constant dry weight was resulted. Moisture content was calculated from the difference between the fresh weight and dry weight and expressed in terms of percent moisture content on the basis of dry weight. The dry weight of the seedlings is expressed in gram per 100 seedlings. All the biochemical estimations were done at 48 h to a period of 10 days. Total sugars, reducing sugars, amino acid content and total protein content in the seedlings were estimated using standard protocols (23-25). All the data were statistically analyzed using ANOVA.

Results and Discussion

Supplementation with growth regulators affected the germination of *T ammi*

A higher percentage of germination was recorded in the seedlings under the substrate GA_3, while lower percentage of germination was noticed with CH (Figure 1A). Initially, both the compounds retarded seedling elongation. After 144 h of germination, seedlings under the substrate GA3 showed greater elongation than the control (Figure 1B). Throughout the period of germination, CH showed inhibitory effect on seedling elongation. All seedlings recorded a gradual increase in moisture content and reached a peak value by 192 h (Figure 1C). Seedlings supplemented with both GA_3 and CH showed lower moisture absorption than the control and a gradual decrease in dry mater content. Both GA_3 and CH positively affected dry matter accumulation compared to the control, while CH being the best (Figure 1D).





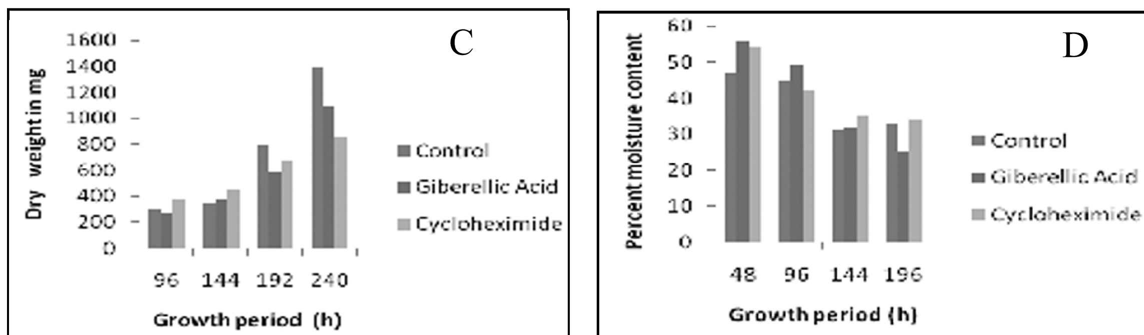


Fig1. A-D : Effect of growth regulators on percentage of seed germination, seedling elongation, moisture content and dry weight of *T. ammi*. All the data were obtained in triplicate treatments and were subjected to the statistical analyses. Analysis of variance (ANOVA) was used to define statistical significance ($P < 0.05$).

Dry seeds recorded low level of reducing sugars. The level of reducing sugars increased as the germination period advanced. The seedlings under the substrates GA_3 and CH showed higher level of reducing sugars compared to the control. Initially the seedlings under the substrate CH showed higher content of reducing sugar as compared to GA_3 substrate. At 192 and 240 h of germination, the seedlings under GA_3 registered higher level of reducing sugars compared to CH. The overall effect of GA_3 and CH indicated that seedlings had considerably higher level of reducing sugars compared to control. The level of reducing sugars was relatively higher in the seedlings under the substrate CH compared to GA_3. At 48 h, there was a low level of reducing sugars, which increased significantly to high level of reducing sugars at 192 h, and decreased to lowest by 240 h of germination (Figure 2A).

In the dry seeds, the level of total sugars was low, and later during germination, the seedlings recorded higher content of total sugars. In the initial stages up to 144 h of germination period the seedlings treated with GA_3 and CH showed lower level of total sugars compared to control. At 192 and 240 h of germination, the seedlings under both the substrates showed higher level of total sugars compared to control. The seedlings under the substrate CH had higher total sugars at 192 h, while the seedlings under the substrate GA_3 had higher total sugars at 240 h. The overall effect of substrates indicated that seedlings under the substrates GA_3 and CH had registered high levels of total sugars than the control. GA_3 caused higher level of total sugars compared to CH (Figure 2B).

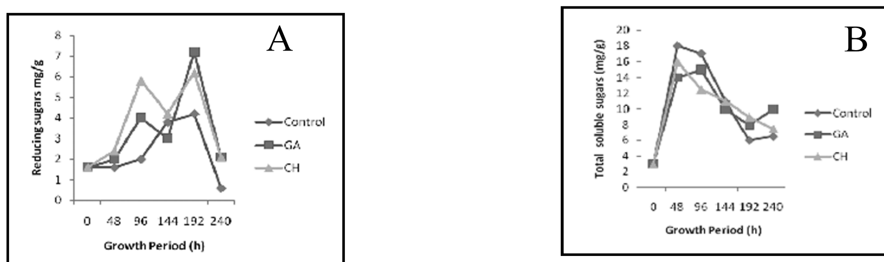


Fig. 2: A-B : Effect of growth regulators gibberellic acid (GA_3) and cycloheximide (CH) on reducing sugars and total soluble sugars in treated seeds of *T. ammi*. All the data were obtained in triplicate treatments and were subjected to the statistical analyses. Analysis of variance (ANOVA) was used to define statistical significance ($P < 0.05$).

The level of total amino acids in all the seedlings increased up to a period of 192 h which finally declined at 240 h of germination. The seedlings under the influence of GA_3 and CH showed higher value of total amino acids at 96 h of germination compared with control seedlings. In all periods, except at 48 h, the level of total amino acids in seedlings under the substrate GA_3 exceeded when compared with those under the substrate CH. The overall effect of substrates indicated that the level of total amino acids was lower in seedlings under the influence of GA_3 and CH, when compared with those of the control. However, the level of total amino acids in seedlings under the influence of GA3 was considerably higher when compared with CH. The level of total amino acids in the seedlings at 240 h was considerably higher when compared to 48 h (Figure 3A).

Dry seeds recorded remarkably low level of total proteins, which increased considerably in the seedlings during the germination periods of 48, 96 and 144 h, while later on the level decreased in other periods and finally attained respective lowest values at 240 h of germination. The seedlings under the substrate CH and GA_3 had higher total proteins almost at all periods compared with control seedlings. The seedlings under the substrate CH showed higher value of total proteins, when compared with those under the substrate GA_3. The overall effect of substrates indicated that seedlings under GA_3 and CH had higher value of total proteins as compared with those of control seedlings. The seedlings under the substrate CH showed highest level of total proteins among all. Studying the effect of periods, the level of total proteins in the seedlings was considerably higher in the initial periods of 48 and 96 h, while later on diminished in other periods and attained the lowest level at 240 h germination period (Figure 3B).

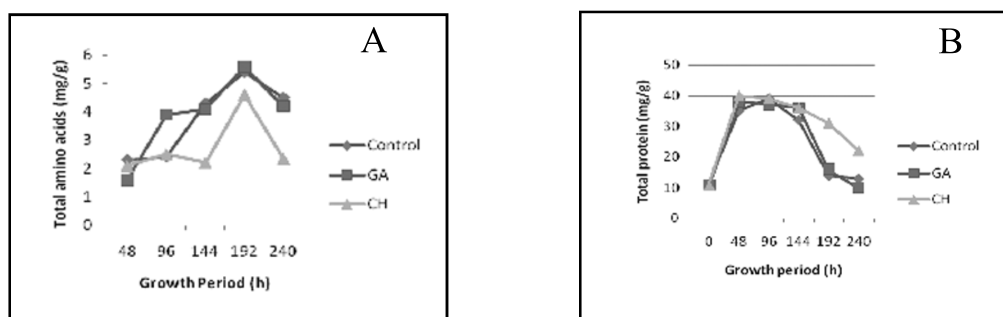


Fig. 3: A-B : Effect of growth regulators gibberellic acid (GA_3) and cycloheximide (CH) on total amino acids and total proteins in germinating seeds of *T. ammi.* All the data were obtained in triplicate treatments and were subjected to the statistical analyses. Analysis of variance (ANOVA) was used to define statistical significance ($P < 0.05$).

Growth regulators and inhibitors have been associated with the control of many physiological processes, including dormancy of seeds and buds, apical dominance, root initiation, flowering, abscission, fruit development, dwarfism and senescence. GA_3 functions as growth promoter by enhancing the activity of hydrolytic enzymes in crop seeds during germination and its influence

on the enzymes of protein and nucleic acid metabolism is well documented (26). Among the various growth inhibitors, CH (actidione) has been the well-studied alicyclic inhibitor which is known to inhibit protein synthesis (26). CH also interferes in cellular respiration and also the uptake of amino acid (27). In the current study, the effect of GA3 on the germination in *T. ammi* indicated that the growth regulator had a growth promoting effect. Such enhancement in germination has been reported earlier in many species viz., Pepper (28), *Momardica charantia* (29), *Capsicum frutescens* (30), *Rhys typhina* (31). CH inhibited the germination process was observed earlier by Chouduri *et al.,* (32), Srivastava *et al.,* (33), Bose *et al.,* (34), and Ramana and Radhakrishnan (35). Reducing sugars in *T. ammi* showed initial increase followed by a gradual decline, whereas the total sugars exhibited sudden increase followed by little decline and little increase in content. Chandra and Banerji (36) also reported decline in reducing sugars during the final stages of germination in mango. A significant increase in total carbohydrate content in the final stages of germination is indicative of the conversion of fats to carbohydrates through the glyoxalate cycle (37). Protein contents in *T. ammi* showed decreasing trend during germination. There was initial increase up to certain stage followed by steep decrease. GA_3 treatment yielded increased amounts of total proteins. The effect of growth inhibitor, CH was also similar to GA_3. The decrease in protein content might be due to the utilization of soluble products in the various physiological processes. This indicates that the products of reserve protein hydrolysis were rapidly translocated to the growing regions of seedlings with the advancement of germination period. Similar observations were reported in pigeon pea (38) and *Adansonia digitata* (39). Free amino acid content was increased in the initial stages which subsequently declined in the later stage. This may be due to the triggering on of protein synthesis in the late stages of germination facilitating greater incorporation of free amino acids in the proteins. It is quite possible that the synthesis of proteins need not necessarily coincide with the onset of seed germination. GA_3 treatment showed slight increase in the content of free amino acids. CH treatment resulted in the initial lowering of amino acid content followed by gradual increase in the later stages. This interesting observation could be attributed to the initial synthesis of protein exhausting the available amino acids. However, in the later stages, CH might have acted upon the translational process obstructing further synthesis of proteins. This, in turn, yielded higher amounts of free amino acids. Alternatively, it could also be due to less utilization of released amino acids by the growing embryo and cotyledon. Webester (40) and Koller *et al.,* (41) have also shown that the decreased content of amino acids is due to further degradation yielding energy to the growing seedlings.

Conclusion

In conclusion it was observed that GA_3 had greatly enhanced the germination in terms of germination percentage, seedling elongation, percent moisture and dry weight. It is interesting to note that GA_3 and CH had stimulatory effect on reducing sugars, total soluble sugars, proteins and amino acids. Total phenols and catalase activity in these seedlings treated with GA_3 and CH are underway in our laboratory.

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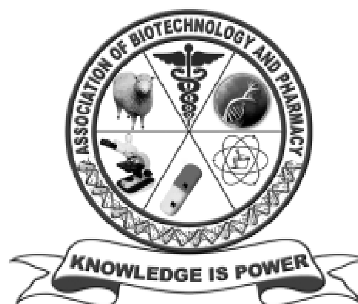

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