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Marslin Gregory **Nanotechnological approaches using curcumin and *Withania somnifera*: neuroprotection and antimicrobial activities**

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Tese de Doutoramento em Biologia de Plantas

Trabalho efectuado sob a orientação do:
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dezembro de 2015

Statement of Integrity

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of my thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 02/12/2015

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Signature: 

A tese de doutoramento aqui apresentada foi desenvolvida no âmbito de financiamento pela Fundação para a Ciência e Tecnologia (FCT) através de uma bolsa individual de doutoramento, com a referência SFRH/BD/72809/2010 (no âmbito do QREN - POPH - Tipologia 4.1 - Formação Avançada, comparticipado pelo Fundo Social Europeu e por fundos nacionais do MCTES).

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Summary/ Sumario

Summary

Neurological disorders contribute to 6.3% of the global burden of the diseases and this number is increasing every year due to aging population. Despite enormous efforts in brain and central nervous system related research, neurodegenerative diseases such as Alzheimer or Parkinson disease remain the world's leading cause of disability. Moreover, neurodegenerative diseases are multifactorial pathogenesis with a complex combination of genetic components and environmental factors. Although, several treatment methods are available to treat these conditions, they are associated with the risk of infection, high neurosurgical cost and limited drug availability due to the presence of blood–brain barrier (BBB).

Herbal medicine for neurological problems has gained much attention in the recent years, because of the disadvantages of conventional therapies and increased patient compliance. Generally, the drugs obtained from natural sources are considered to be safe alternatives. Since ancient times, plant extracts have been used in the treatment of pathologic conditions of the central nervous system. As the methods to isolate active constituents from plant extracts have greatly improved, the scientific understanding of psychoactive plants has also advanced significantly. Curcuma species and Withania somnifera have been used as traditional therapeutic agents in Asian medicine to treat various common ailments and recently reported to possess antioxidant, antidepressant and neuroprotective effects.

Although a number of herbal-based medicines have been screened and identified, still the treatment for the neurological disorders is not up to the required level. This is mainly because most of the herbal medicines exhibit poor bioavailability due to low absorption, fast metabolism, and rapid systemic elimination in the body. Although several approaches have been employed to overcome these problems, modification in the delivery method is a promising approach. In particular, employing herbal medicine based nanocarrier system to treat neurological disorder has gained specific interest because of its several advantages namely, the ability of nanoparticles to deliver the drug in a pre-determined rate at a particular site of action, providing high bioavailability and low toxicity. Compared to the conventional delivery methods, nanoparticle-mediated administration of drugs has been proven to be the ideal way to deliver drugs for neurological disorders. Due to these advantages, pharmaceutical scientists have started designing nanoparticle mediated drug delivery systems for herbal medicines with neurotherapeutic

potential. Nanoencapsulation of bioactive compounds and extracts into the polymer matrix gives several advantages, including protection against degradation, enhancing the solubility and bioavailability.

In this PhD thesis we have selected two important medicinal plants with proven antioxidant, antidepressant and neuroprotective effects namely *W. somnifera* and *Curcuma* sps for nanoencapsulation and subsequent cellular uptake with U251 glioma cells and biodistribution analysis in Zebrafish model.

First we developed HPLC methods to identify withanolides and quantify curcumin respectively from *W. somnifera* extracts and *Curcuma* species. Extracts derived from different parts (leaves, roots and fruits) of *W. somnifera* were tested. *W. somnifera* extract with maximum amount of withanolides and curcumin (active principle of *Curcuma* sps) were selected for nanoencapsulation. We found that Purospher RP-18 e 5 µm column is ideal for efficient separation and simultaneous quantification of withanolides whereas, Purospher C-18 column was optimum for curcumin quantification from *Curcuma* sps extracts. HPLC quantification of different extracts of *W. somnifera* revealed that the methanolic extract obtained from leaf tissues contain the highest amount of withanolides compared to roots and fruits. Compared to *C. aromatica*, *C. longa* possess higher amount of curcumin.

W. somnifera extract/curcumin loaded polycaprolactone (PCL) and methoxy polyethylene glycol- polycaprolactone (MPEG-PCL) nanoparticles were prepared by solvent displacement method. Prepared nanoparticles were characterized for their physico-chemical properties such as size, shape, *in vitro* drug release and encapsulation efficiency. After the physico-chemical characterization, we analyzed the *in vitro* cytotoxicity and cellular uptake of nanoparticles by U251 glioma cells. In addition, we evaluated the protective effect of *W. somnifera* extract and curcumin loaded nanoparticles against tBHP-induced insult in U251 glioblastoma cells, as a measure of neuroprotection. Biodistribution of *W. somnifera* /curcumin loaded PCL and MPEG-PCL nanoparticles in animal system were evaluated using zebrafish larvae as model organism.

Physical characterization of the prepared nanoparticles revealed that MPEG-PCL nanoparticles were smaller in size compared to PCL nanoparticles irrespective of *W. somnifera* extract or curcumin encapsulation. Transmission scanning electron microscopy (TEM) images of nanoparticles revealed a round shape in general, although the surface of PCL nanoparticles appeared to be rough and porous. The entrapment efficacy of *W. somnifera* extract and curcumin was higher in MPEG-PCL compared to PCL. We observed an initial burst release followed by a slow

extended release profile for both *W. somnifera* extract and curcumin irrespective of the polymer used for encapsulation.

Treatment of U251 glioma cells with PCL and MPEG-PCL nanoparticles loaded with *W. somnifera* extract/ curcumin evidenced the efficient cellular uptake of nanoparticles. However, MPEG-PCL nanoparticles showed better internalization compared to PCL nanoparticles, irrespective of their *W. somnifera* extract/ curcumin load. Neuroprotection assay showed that both *W. somnifera*/ curcumin loaded nanoparticles protect the cells from oxidative damage. While the neuroprotective effect of *W. somnifera* extract increased in a dose dependent manner, curcumin was much effective in lower concentrations. Together, our results show that *W. somnifera*/ curcumin loaded MPEG-PCL nanoparticles possess significantly higher neuroprotective effect in U251 human glioma cells compared to the free drugs and their PCL counterparts.

The *in vivo* localization of nanoparticles in Zebrafish model suggested that the MPEG-PCL nanoencapsulation had efficient and quicker delivery into the larvae compared with the free drug or PCL nanoparticles. Fluorescence imaging of nanoparticles revealed that the nanoparticles were distributed throughout the animal. However, in terms of fluorescence, the head region of animals treated with MPEG-PCL nanoparticles was more intense than that of PCL nanoparticles, indicating that the former might be accumulating in the brain region. Together our results suggest that delivery of *W. somnifera* extract/ curcumin as MPEG-PCL nanoparticles could enhance the neuroprotective activity.

We have also tested the possibility of green synthesis of silver nanoparticles using *W. somnifera* extract and evaluated the biosynthesized silver nanoparticles for their antibacterial activity in a cream formulation. We developed a simple, fast and cost effective green synthesis technique for silver nanoparticles with potent antimicrobial activity using *W. somnifera* extract. We also found that catechin, *p*-couparic acid, luteolin-7-glucoside and a non-identified withanolide are the various compounds present in *W. somnifera* aqueous leaf extract responsible for green synthesis. The cream incorporated with silver nanoparticles showed antimicrobial activity against a wide variety of clinical isolates.

Sumario

As doenças neurodegenerativas representam 6,3% do gasto geral na saúde e este número tem vindo a aumentar continuamente, anualmente, devido ao envelhecimento da população. Apesar do imenso esforço feito na investigação de doenças foro neurológico, as doenças neurodegenerativas, tais como Alzheimer ou Parkinson, permanecem uma das maiores causas de incapacidade em humanos. Além disso, as doenças neurodegenerativas paresentam patogénese multifactorial, com uma complexa combinação de componentes genéticos e factores ambientais. Apesar de existirem alguns métodos usados no tratamente destas patologias, elas estão associadas a risco de infecção, custos elevados e biodisponibilidade reduzida devido à presença da barreira hematoencefálica (BEE).

O uso de plantas medicinais em patologias do foro neurológico tem sido bastante estudado nos anos recentes, em virtude das desvantagens da terapia convencional e indo ao encontro da vontade dos pacientes. De um modo geral, os produtos farmacológicos derivados de plantas são considerados alternativas seguras. Desde tempos remotos que o uso de extractos de plantas tem sido utilizado no tratamento de patologias do foro neurológico. Assim como os dos métodos de extracção dos compostos de plantas têm evoluído significativamente, também o conhecimento científico da acção destes compostos a nível neurológico tem aumentado de forma substancial. A curcuma e *Withania somnifera* são utilizadas na medicina tradicional asiática no tratamento de várias patologias e recentemente foram evidenciadas as suas actividades antioxidante, antidepressiva e neuroprotectora.

Apesar de algumas formulações, compostos e fitofármacos baseados em plantas terem sido identificados como potenciais no tratamento de várias neuropatologias o seu desenvolvimento ainda não está no nível requerido para a sua utilização. Uma das razões principais deve-se ao facto deste exibirem baixa biodisponibilidade devido à sua baixa absorção, metabolismo e eliminação rápida pelo corpo humano. Comparado com os métodos convencionais, a administração de fármacos via nanoparticulas tem provado ser uma alternativa eficaz para o uso em neuropatologias. Devido a estas vantagens, os pesquisadores têm vindo a desenvolver sistemas baseados em nanopartículas como veículos de extractos e compostos de plantas com actividade neurológica reconhecida, para o uso em neuropatologias. A nanoencapsulação de compostos e extractos bioactivos numa matriz polimérica apresenta várias vantagens, nomeadamente protecção contra a degradação, aumento da solubilidade e biodisponibilidade.

Neste trabalho foram seleccionadas duas plantas medicinais importantes, com actividade antioxidante, antidepressivo e efeitos neuroprotetores comprovados, designadamente *W. somnifera* e *Curcuma* sp., para nanoencapsulação e posteriores estudos em células U251 (de glioma) e análise de biodistribuição no modelo do peixe zebra.

Metodologias de HPLC foram desenvolvidas para identificar a curcumina (princípio activo de *Curcuma* sp.) e whitanólidos presentes em extractos de *Curcuma* sp e *W. somnifera*, respectivamente. Extractos obtidos de diferentes partes (folhas, raízes e frutos) de *W. somnifera* foram testados. Os extractos contendo a máxima quantidade de withanólidos e curcumina foram seleccionados para nanoencapsulação. Identificou-se que a coluna Purospher RP18 (5□m) é a ideal para uma separação eficiente e a quantificação simultânea dos withanólidos e para uma eficiente quantificação de curcumina em extractos de *Curcuma sp.*. A quantificação por HPLC de diferentes extratos de *W. somnifera* revelou que o extrato metanólico obtido a partir de tecidos foliares contêm a maior quantidade de withanólidos, por comparação com raízes e frutos. Em comparação com *C. aromatica*, a *C. longa* possui maior quantidade de curcumina.

Extractos de W. somnifera / curcumina foram incorporados em nanopartículas de policaprolactona (PCL) e polietilenoglicol metoxi-policaprolactona (MPEG-PCL) usando o método de deslocamento de solvente. As nanopartículas preparadas foram caracterizado pelas suas propriedades físico-químicas, tais como tamanho, forma, libertação do fármaco e a eficiência de encapsulação. Após a caracterização físico-química, analisou-se a citotoxicidade in vitro e a absorção celular das nanopartículas usando células de glioma U251. Além disso, avaliou-se o efeito protector das nanopartículas contendo extracto de W. somnifera ou curcumina contra o insulto oxidativo induzida por t-BHP, como uma medida de neuroproteção. A biodisponibilidade destas nanopartículas contendo o extracto de de W. somnifera ou curcumina foi avaliada ainda num modelo animal, usando alevins de peixe zebra.

A caracterização física das nanopartículas revelou que as nanopartículas de MPEG - PCL foram menores em dimensão em comparação com as nanopartículas de PCL, independentemente do encapsulamento ter utilizado extracto de W. somnifera ou curcumina. A microscopia de varrimento electrónico de transmissão (TEM) das nanopartículas revelou uma forma redonda, em geral , embora a superfície das nanopartículas de PCL parece ser áspero e poroso.

A eficácia de aprisionamento do extracto de W. somnifera e curcumina foi maior em MPEG-PCL, quando comparado com o PCL. Observou-se uma libertação rápida. inicial, seguida de um perfil de libertação lenta e prolongada para ambos extractos, W. somnifera e curcumina, independentemente do polímero utilizado para a encapsulação.

O tratamento de células U251 (giloma) com nanopartículas de PCL e MPEG-PCL carregadas com extracto de W. somnifera ou curcumina evidenciou uma absorção celular eficiente de nanopartículas. No entanto, as nanopartículas de MPE-PCL mostraram uma melhor internalização em comparação com as nanopartículas de PCL, independentemente da sua carga com extracto de W. somnifera ou curcumina. Ensaios de neuroproteção mostraram que nanopartículas contendo extracto de W. somnifera ou curcumina protegem as células dos danos oxidativos. Enquanto o efeito neuroprotector do extracto W. somnifera aumentou de uma forma dependente da dose, a curcumina foi muito eficaz em concentrações mais baixas. Em conjunto, os nossos resultados mostraram que em células de glioma humano U251 as nanopartículas de MPEG-PCL carregadas com extracto de W. somnifera ou curcumina possuem, significativamente. um maior efeito neuroprotector em comparação com os fármacos livres ou seus homólogos encapsulados em PCL .

A localização in vivo das nanopartículas no modelo de peixe zebra sugeriu que a nanoencapsulação em MPEG-PCL teve uma biodisponibilidade mais eficiente e rápida quando comparado com o fármaco livre ou em nanopartículas de PCL. Imagiologia de fluorescência das nanopartículas revelou que as mesmas encontram-se distribuídas por todo o animal. No entanto, a região da cabeça dos animais tratados com nanopartículas de MPEG-PCL era mais intensa do que com nanoparticulas de PCL. Os resultados sugerem que a entrega de extracto de W. somnifera / curcumina por nanopartículas de MPEG-PCL poderá aumentar a atividade neuroprotetora.

Também foi testada a possibilidade de biossíntese de nanopartículas de prata usando o extrato de W. somnifera e avaliou-se a sua atividade antibacteriana numa formulação de creme. Foi desenvolvido um método rápido, de baixo custo e eficaz para a síntese verde de nanopartículas de prata com potente atividade antimicrobiana utilizando o extrato de W. somnifera. Também foi evidenciado que entre os vários compostos presentes no extracto de W. somnifera, a catequina, ácido p-couparico, luteolina -7- glicosídeo e um withanolido (não

identificada) são os responsáveis pela biossíntese das nanopartículas. O creme incorporando nanopartículas de prata mostrou actividade antimicrobiana contra uma ampla variedade de isolados clínicos (bactérias e leveduras).

Publications

Publications

Some part of the thesis has been published or submitted for publication in international peer-reviewed journals:

Marslin G, Selvakesavan R.K, Franklin G, Bruno Sarmento, Alberto C.P. Dias (2015) Antimicrobial activity of cream incorporated with silver nanoparticles biosynthesized from Withania somnifera. International Journal of Nanomedicine 10, 5955–5963. http://doi.org/10.2147/IJN.S81271.

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Marslin G, Bruno Sarmento, Alberto C.P. Dias. (2014) Nano encapsulation of a Withania somnifera extract with PCL and MPEG-PCL di-block copolymer. Planta Medica DOI: 10.1055/s-0034-1394832.

Marslin G, Selvakesavan RK, Franklin G, Bruno Sarmento, Alberto CP Dias. (2014). Green synthesis of silver nanoparticles using *Withania somnifera* aqueous extract incorporated silver nanoparticles for antibacterial activity. Planta Medica. DOI:10.1055/s-0034-1394514

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List of abbreviations

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AD	Alzheimer Diseases
AFM	Atomic Force Microscope
BBB	Blood-Brain Barrier
CV	Cell viability
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Desoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSC	Differential Scanning Calorimeter
EA	Ethyl acetate
FT-IR	Fourier Transform Infrared Spectroscopy
GBM	Glioblastoma Multiforme
HPLC-DAD	High performance liquid chromatography diode array detection
IP	Indian Pharmacopeia
IV	Intra venous
µg	microgram
mg	milligram
ml	milliliter
MPEG	Monomethyl poly(ethylene glycol)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance Spectroscopy
NP	Nanoparticles
PBS	Phosphate Buffer Saline
PCL	poly caprolactone
PDI	Polydispersity index
r^2	Coefficient of determination
RMT	Receptor-mediated endocytosis
Rpm	Rotations per minute
SLP	Solid Lipid Nanoparticles
ŧBHP	Tert-butylhydroperoxide
TEM	Transmission Electronic Microscope
UV	Ultra Violet
WS	*Withania somnifera*
WSE	*Withania somnifera extract*

Chapter 1:
General Introduction

1. General Introduction

1.1 Medicinal plants

Plants are being used as medicines throughout the human history. Even wild animals instinctively eat certain plants to treat themselves certain illnesses. Plant products are one of the major sources of drugs and drug leads (Brusotti et al., 2014) as evidenced by a large number of impressive modern drugs have been derived from natural sources particularly from plants (Pan et al., 2013). In fact, many of these drugs were developed based on their traditional uses medicines. The practice of herbal medicine is extremely well established and documented in Asia (Zhang et al., 2011).

Plants produce and accumulate different types of compounds. Molecules with an important role in basic life functions such as cell division, growth, respiration, storage and reproduction were described as primary metabolites (Bougard et al., 2001). On the other hand, several other compounds which are not essential for the above functions are known as secondary metabolites. For a long time secondary metabolites have been considered as waste by-products of plant metabolism. Recent improvement of biochemical techniques and the rise of molecular biology have shown that these molecules play a major role in the adaptation of plants to their environment including biotic and abiotic challenges. They also act on animals (anti-feeding properties), pathogens (phytoalexins) and other plants (allelopathy) (Bougard et al., 2001). Plant secondary metabolites include a vast array of compounds that to date sum up to more than 200,000 defined structures.

Today, the trend in healthcare is moving towards disease prevention rather than treatment, wherein people choose herbal medicines, functional foods and nutraceuticals over pharmaceuticals for their wellbeing (Brower et al., 2005). There is a shift in universal trend from synthetic to herbal medicine, which can be said “Return to Nature”. Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of diseases and ailments. In general, people believe that medicinal plants are more natural and more easily accessible than manufactured drugs (Petrovska et al., 2012). This drift has brought more market opportunities for herbal products, as evidenced by the widespread usage of natural products among the therapeutics used in medical treatment. Spurred by the increasing aging population and consumer awareness about general health and wellbeing, the global herbal supplements and remedies market (GHSRM) exhibited significant growth over the last decade, evidenced by the rapid expansion of plant-based

pharmaceutical industries and the increasing demand for medicinal plants. The market research report on herbal supplements and remedies accounts Europe as the largest regional market of the entire world, which is followed by Asia-Pacific and Japan.

Studies on medicinal plants are rapidly increasing in order to search for new active molecules or to improve their production for the herbal pharmaceutical industries (Poutaraud and Girardin, 2005). A large number of plants have been found to contain ingredients that possess antibacterial, antifungal, and anticancer activities, while others are recognized for their antioxidant properties. Many bioactive compounds present in plant extracts are too complex to be chemically synthesized. Of the 175 small molecules developed from approximately the 1940s to date, 131 (74.8%) are non-synthetic. Among these, 85 (48.6%) are either genuine natural products or derived directly from natural products (Newman and Cragg, 2012). In some therapeutic areas, the contribution of natural products is even greater, e.g. about 78% of antibacterial and 74% of anticancer drug candidates are natural products or structural analogues of natural products. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancers were of natural origin. Recently some products of higher plant origin have been shown to be effective sources of chemotherapeutic agents without undesirable side effects. More than two thirds of anticancer drugs approved between the 1940s and 2006 are natural products or were developed on the basis of natural products. Well known examples include Vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), epipodophyllotoxines (etoposide, teniposide), camptothecine and its derivatives (topothecan, irinothecan), and anthracyclines (doxorubicin, daunorubicin,epirubicine, idarubicin).

1.2. *Withania somnifera*

Withania somnifera (WS), commonly known as *Ashwagandha*, is an important plant in Indian medicine. This species is distributed in the dry parts of India, Baluchistan, Pakistan, Afghanistan, Sri Lanka, Congo, South Africa, Egypt, Morocco, and Jordan (Kulkarni and Dhir, 2008). It is a green shrub and belongs to Solanaceae family. It has several vernacular names including *Indian ginseng* and *winter cherry* (Andallu and Radhika, 2000)., In India, *W. somnifera* is cultivated and either its whole plant extract or separate constituents have been used in ayurvedic and indigenous medicine for over 3000 years. Although only the root (known as *Asgand*) of this plant was originally used in Unani medicine, the medicinal potential of its leaves and fruits were also identified sooner and adapted in Indian traditional medicine. Currently, different forms (decoction, infusions, ointment powder and

syrup) of different parts (leafs, roots, fruits) of this plant are used as medicine all over the world (Gregory et al., 2014a; Gregory et al., 2014b). *W. somnifera* is commonly available as a fine sieved powder that can be consumed by mixing in water, ghee (clarified butter) or honey.

The major pharmacological activities of *W. somnifera* is due to its adaptogenic, anticonvulsant, antisedative, anticarcinogenic, immunomodulatory and antioxidative properties (Mishra et al., 2000; Ven Murthy et al., 2010;) Steroidal lactones called withanolides are the major active compounds present in *W. somnifera* (Dhar et al., 2006; Misra et al., 2008). More than 40 withanolides (a group of C28 steroids characterized by a 6-membered lactone ring in the 9-carbon atom side chain), 12 alkaloids, and several sitoindosides have been isolated from different parts (leaves, roots and cherries) of *W. somnifera.* The medicinal uses of different parts of *W. somnifera* are summarized in the following table.

Table 1. Medicinal uses of different parts of *W. somnifera*

W. somnifera **Plant parts**	**Medicinal uses**	**Reference**
Leaf	*W. somnifera* extract act as a free radical scavenger, protecting cells against toxic insult	(Kumar et al., 2014)
	Water extract of *W. somnifera* extract poses anti-cancer activity in in-vitro and in-vivo models	(Wadhwa et al., 2013)
	Anti-oxidant and anti-bacterial activity	(Alam et al., 2012)
	W. somnifera showing great antimicrobial potential against test microorganisms	(Singh and Kumar, 2011)
	Anti-proliferative and anti-oxidative activities of *W. somnifera* leaf extract from *in vivo* and *in vitro*	(Kaur et al., 2004)
	Phenolic acids and flavonoids are responsible for anti-inflammatory activity	(S. Sivamania, 2014).
	Amnesia and its recovery by alcoholic extract of *W. somnifera*	(Gautam et al., 2013)
	Potential for prevention of neurodegeneration associated with glutamate-induced excitotoxicty	(Kataria et al., 2012)
	W. somnifera leaf extract serve as potential preventive and therapeutic agents for neurodegenerative disorders	(Konar et al., 2011)
	W. somnifera leaf extract protects cells from MAA-induced toxicity by suppressing the ROS levels, DNA and mitochondrial damage	(Priyandoko et al., 2011)
	Anti-diabetic activity of *W. somnifera* leaf extract	(Gorelick et al., 2015)
	Molecular insight into the immune up-regulatory properties	(Khan et al., 2009)
Fruits	Oxidative and inflammatory	(Ojha et al., 2014)

	Blood–brain barrier permeability of withanolides in *W. somnifera* fruit extract	(Vareed et al., 2014)
	Potent lipid peroxidation inhibitors effect *W. somnifera* fruits	(Jayaprakasam et al., 2004; Jayaprakasam et al., 2010)
	Anti-cancer Properties	(Oza et al., 2010)
	Diuretic and hypnotic	(Saritha and Naidu, 2007)
Roots	The root extract of *W. somnifera* is rich in several components with strong antioxidant properties	(Bhattacharya et al., 1997; Dhuley et al., 1998)
	W. somnifera root extract is used as a dietary supplement	(RajaSankar et al., 2009)
	W. somnifera root is used as a Rasayana in the Indian ayurvedic tradition	(Khan et al., 2015)
	Aqueous extract of *W. somnifera* exhibited antioxidant and anti-arthritic activity and reduced inflammation	(Khan et al., 2015)
	Aqueous extract of *W. somnifera* exhibited antioxidant and anti-arthritic activity and reduced inflammation	(Khan et al., 2015)
	Root extract reduced the number of mammary carcinomas that developed and reduced the rate of cell division in the carcinomas	(Khazal et al., 2014)
	Root extract significantly reduced the rate of cell division in the mammary tumors	(Khazal et al., 2013)
	Spermatogenic activity	(Ambiye et al., 2013)
	Anti-inflammatory effect of *W. somnifera* root	(Gupta and Singh, 2014)
	Stress and anxiety	(Chandrasekhar et al., 2012)
	Neuroprotective	(Prakash et al., 2013)
	Root extract of *W. somnifera* protected the adverse effects of LN intoxication in mice	(Sharma et al., 2012)
	W. somnifera root extract protect neurodegeneration	(Baitharu et al., 2013)
	Scalp hair loss	(Kalani et al., 2012)
	Aqueous extract of *W. somnifera* roots has an ability to inhibit the formation of mature amyloid-β fibrils in vitro	(Kumar et al., 2012)
	Antiinflammatory and muco-restorative activity	(Pawar et al., 2011).
	Enhancing the antioxidant system and protecting the cellular integrity of kidney and liver tissues	(Jeyanthi and Subramanian, 2010).
	Immunologic effects	(Mikolai et al., 2009).
	Anxiety and depression	(Gupta and Rana, 2007)
	Anti-cataleptic effect	(Nair et al., 2008)
	W. somnifera root extract have the anti-oxidant and anti-arthritic activity	(Khan et al., 2015)

1.3. *Curcuma sps*.

Curcuma is a genus of about 100 species in the family Zingiberaceae. Rhizomes of the *Curcuma longa* and *Curcuma aromatica* species is known as turmeric and used in India as traditional medicine as well as cosmetic product. Although both species exhibits medicinal values, *C. aromatica* is mainly used in cosmetic and facial products. The bright yellow color present in the turmeric is mainly due to curcumin, a polyphenolic, highly potent, nontoxic bioactive compound found in turmeric. Recent research suggests that curcumin may possess a broad range of pharmacological activities such as antioxidant, antiinflammatory, antimicrobial, antiviral, antirheumatic, and neuroprotective properties. Among the above pharmacological properties, anticancer and antioxidant activities of curcumin are well-documented. Important medicinal uses of *C. longa* and *C. aromatica* are summarized below (Table 2).

Table 2. Medicinal uses of *C. longa* and *C. aromatica*

Species	Uses	Reference
C. aromatica	Antibacterial activity	(Revathi and Malathy, 2013)
	Antiproliferative and apoptotic	(Dai et al., 2013)
	To treat colon carcinoma	(Hu et al., 2011)
	Antioxidant activity	(Al-Reza et al., 2010)
	Antitussive activity	(Marina et al., 2008)
	Chemoprotective effect	(Li et al., 2009)
	Hepatoma cell proliferation inhibitory effects	(Wu et al., 2000)
	Antiinflammatory effect of the volatile oil from	(Li, 1985)
C. longa	Wound healing	(Pawar et al., 2015)
	Antiinflammatory Activity	(Illuri et al., 2015)
	Antiarthritis	(Nonose et al., 2014)
	Inflammatory bowel disease	(McCann et al., 2014)
	Antiglycation and antioxidation properties	(Khan et al., 2014)
	Antidiabetic activity	(Ahmad et al., 2014)
	Hepatoprotective	(Sengupta et al., 2011; Kim et al., 2014)
	Antidepressant	(Xia et al., 2007; Yu et al., 2002)
	Antiviral	(Kim et al., 2009)
	Antifertility effect	(Mishra and Singh, 2009)

	Neuroprotective	(Issuriya et al., 2014)
	Immunostimulatory effect	(Yue et al., 2010)
	Antioxidant	(Singh et al., 2010)
	Activity against UVB irradiation-induced skin damage	(Sumiyoshi and Kimura, 2009)
	Anticonvulsant effect	(Orellana-Paucar et al., 2012)
	Antiplatelet property	(Lee, 2006)
	Cardioprotective	(Mohanty et al., 2004)

1.4. Neurological disorders

Neurological disorders are a major contributors of the global burden of the diseases, which is increasing every year due to aging population (Patel et al., 2012). Despite enormous advances in brain research, brain and central nervous system disorders remain the world's leading cause of disability. Neurodegenerative diseases such as Alzheimer and Parkinson are multifactorial pathogenesis with a complex combination of genetic components and environmental factors. Although several treatment methods are available to treat this conditions, they are associated with the risk of infection, high cost and limited drug availability (Hu et al., 2009). The lack of efficiency in the treatment is mainly due to the presence of blood–brain barrier (BBB) that protects the central nervous system from exogenous toxicants, but it also restricts the passage of potential neuro therapeutic compounds. As a result the availability of the drug concentration in the brain is insufficient to treat brain disorders.

In spite of significant efforts to elucidate the causative factors, the pathogenesis of these diseases is still unclear. However, excitotoxicity is considered as a common pathologic event underlying neurodegeneration in several neurodegenerative diseases, (Kang et al., 2005). Additionally, oxidative stress is also regarded as a factor contributing to the development of neurodegenerative diseases (Behl and Moosmann, 2002). However, whether it is a causative factor or a consequence of neuronal death is presently not clear. Together with apoptosis, these events seem to underlie the specific neuronal loss affecting areas associated with cognitive functions and memory, as in Alzheimer's disease and other forms of dementia (Pereira et al., 2005; Tannenberg et al., 2006) or motor functions and speech, as in Parkinson's and Huntington's diseases (Barzilai and Melamed, 2003).

1.5. Treatment strategies for neurological disorders

1.5.1. Herbal medicine for neurological disorders

The initial drugs used in the treatment of pathologic conditions of the central nervous system (CNS) were based on natural resources, especially plants. The scientific understanding of psychoactive plants has advanced significantly after the isolation of active constituents, such as morphine from the *Opium poppy* species (Gomes et al., 2009). *Hypericum perforatum* has been recognized in the treatment of mild to moderate depression and many cases; it has been better tolerated than conventional antidepressants (Kasper et al., 2010). Curcumin, the natural compound obtained from *C. longa* has been used as a traditional therapeutic agent in Asian medicine to treat various common ailments. Recently it has been reported to possess antioxidant, antidepressant and neuroprotective effects (Issuriya et al., 2014;Zaky et al., 2014). The phenolic compounds of *Z. piperitum* leaf are utilized as an effective and safe functional food containing natural antioxidants, reducing the risk of neurodegenerative disorders (Jeong et al., 2011). Use of herbal medicine for neurological disorders has gained much attention in the recent years, because of the disadvantages of conventional therapies and increased patient compliance. In general, drugs obtained from natural sources are considered to be safe alternatives for the treatment of several diseases including neurological disorders (Katiyar et al., 2012).

1.5.2. Drawbacks of herbal medicines in the treatment

Although a number of herbal medicines have been identified, they are not up to the level for being used in the treatment of neurological disorders. This is mainly due to the fact that most of the herbal medicines exhibit poor bioavailability due to poor absorption, fast metabolism, and rapid systemic elimination. To overcome these problems, several approaches have been employed and among them, modification in the delivery method is a promising approach (Tsai et al., 2011). In particular, herbal medicine based nanocarrier system to treat neurological disorder has gained specific interest because of several advantages such as the ability of nanoparticles to deliver the drug in a pre-determined rate at a particular site of action, providing high bioavailability and low toxicity (Ajazuddin and Saraf, 2010). Compared to the conventional delivery methods, nanoparticle-mediated drug delivery has been proven to be the ideal way to deliver drugs for neurological disorders. For instance, Ray et al. (2011) found that the neuroprotective effect of curcumin was increased when administered as polymeric nanoparticle (Ray et al., 2011). Similarly, the efficiency of quercetin to treat Alzheimer´s disease was

also significantly enhanced when it was delivered to the brain as solid lipid nanoparticles (Dhawan et al., 2011). Knowing the advantages and the upcoming trend, pharmaceutical scientists have started designing nanoparticle mediated drug delivery systems for herbal medicines with neuro therapeutic potential.

1.5.3. Transport mechanism of molecules across the blood brain barrier (BBB)

The treatment for neurological disorders such as schizophrenia, meningitis, migraine, Parkinson's disease and Alzheimer's disease require the drug to be delivered to the brain at a particular concentration, over a period of time (Laquintana et al., 2009). But the transport of the drug molecules to the brain is restricted by the defense mechanism of the brain called the blood brain barrier (BBB). Although, this is the life supporting protection of the brain, it also eliminates most of the drug from reaching brain, making it an important issue to be considered while designing novel drug delivery strategies (Temsamani et al., 2000).

The mechanism of transcellular diffusion of molecules through the BBB depends on their structural and physicochemical properties, such as molecular size, charge, lipophilicity and hydrogen bonding potential. Approximately, 98% of small molecule drugs and almost all large molecule drugs (mw>1kDa) do not cross BBB (Krishnamoorthy et al., 2014). The transport mechanism of molecules across the BBB is schematically represented in **Figure 1**.

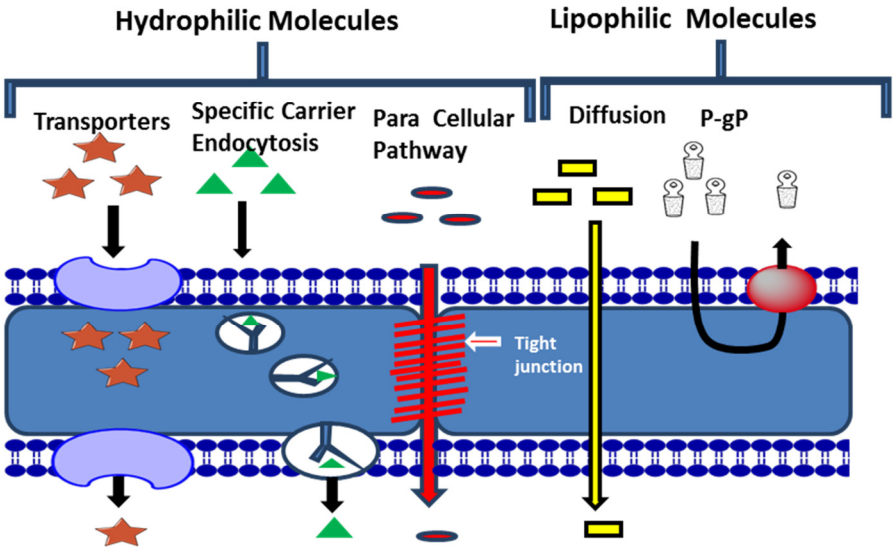

Figure 1. Schematic representation showing the transport of drug molecules across the BBB (Adapted from (Gabathuler, 2010)) Small lipophilic drugs may enter by penetrating the luminal and abluminal membranes by passive diffusion. Non-diffusible molecules can cross the endothelial cells via active carrier-mediated transport.

Apart from certain compounds that are essential for brain homeostasis, such as amino acids, glucose, neuropeptides, and proteins which are transported into the brain via specific endogenous blood-brain transporters, only small lipophilic molecules with molecular weight below 400Da are able to cross this complex barrier by transcellular diffusion. Even such molecules should not bind to plasma proteins in a high level or form a substrate for a transport system at the BBB (Gabathuler, 2010). If the molecular weight of a drug molecule is higher than 400Da or the drug forms more than eight hydrogen bonds, the probability of crossing the BBB via transcellular diffusion, in pharmacologically significant amounts is very low. To allow access for various molecules that are essential for brain maintenance and function, specific endogenous blood - brain barrier transporters (carriers or receptors) exist in the luminal and basolateral membranes of the endothelium. Carriers are membrane-restricted systems commonly involved in the transport of small molecules with a specific size and a molecular weight smaller than 600Da. Carrier mediated transcytosis is used in the transport of nutrients to the brain and only drugs with specific characteristics that closely mimic the endogenous carrier substrates will be internalized.

1.5.4. Common strategies of drug delivery to the brain

Delivery of drugs across the BBB to treat neurological disorders has become a major concern due to the poor permeability of therapeutic agents. Hence, various strategies have been introduced to overcome the above-mentioned problem (**Figure 2;** Alam et al., 2010).

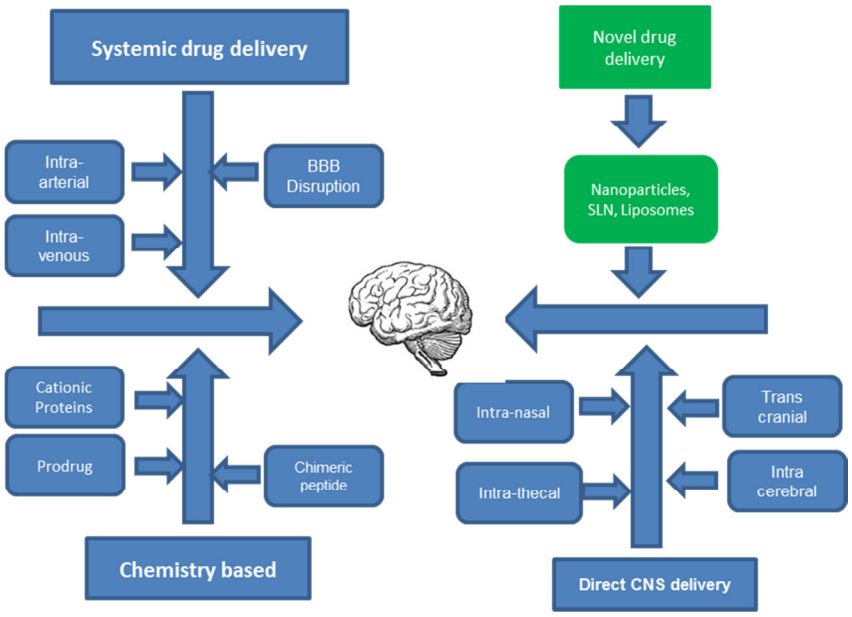

Figure 2. Schematic representation mentioning various approaches through which, the drug can be targeted to the brain.

1.5.5. Systemic drug delivery

In systemic drug delivery method BBB is temporarily broken down by injecting a sugar solution like mannitol into arteries of the neck. The high sugar concentration in brain capillaries sucks water out of the endothelial cells, shrinking them thus opening tight junctions (Hartz et al., 2005) . There are three different techniques employed to disrupt tight junctions: (i) osmotic disruption; (ii) MRI –guided focused ultrasound BBB disruption; (iii) Application of bradykinin analogue (Etame et al., 2012). But these techniques are used only to temporarily deliver the chemotherapeutic agents in patients. Also these techniques allow the drugs to enter even normal cells, causing unwanted side effects. Moreover, these methods require hospitalization and anesthesia and also have the possibility of causing neuron damage.

Alternatively, the drug can be delivered to the brain intravenously. However, since this is a non-site specific drug delivery that is subjected to rapid clearance from extracellular fluid, a large dose of drug is administered, which is the main demerits of this method. Chemistry based drug delivery is site specific delivery of the drug through multistep enzymatic and chemical modification. Mainly the chemical method includes pro drug and drug conjugate. The permeability of the drug can be increased by the chemical modifications (Misra et al., 2003).

Intra-nasal administration is also applied to deliver the drug in the brain, where, direct movement of the drug from sub mucosa space of the nose to the brain and cerebrospinal fluid can be achieved (Pires et al., 2009). Intranasal drug administration offers rapid absorption to the systemic blood avoiding first-pass metabolism in the gut wall and the liver. Several studies have shown a direct route of transport from the olfactory region to the CNS in animal models, without prior absorption to the circulating blood. Since drugs absorbed via the olfactory route do not have to cross the BBB it may be possible to deliver substances to the CNS that would otherwise have been blocked from entering via the systemic circulation. Through intrathecal injection, the drug can be administered directly into cisterna manga of the brain. It is less invasive compared with intra-ventricular administration. This route of administration is better for the treatment for spinal diseases. However, there is a chance of drug spreading along the distal space of spinal canal (Miyake and Bleier, 2015).

1.5.6. Biological methods

The biological method includes the use of monoclonal antibodies, vectors or receptors. Here the main idea is to conjugate the drug with antibodies and the conjugate can then be directed towards an antigen residing on or within the target tissue, enabling the drug to reach the brain by receptor-mediated transcytosis mechanisms (Garcia-Garcia et al., 2005). OX26, 8D3 MAb or R17 217 MAb are antibodies for transferrin receptor (TfR), which allowed the conjugated drug to undergo receptor-mediated transcytosis across the BBB of mouse (Pardridge, 2002).

1.5.7. Nano based drug delivery systems for the brain

A promising novel approach to deliver drugs to the targets within the CNS is the employment of colloidal carriers. Several reviews have been published and discussed the advantages and disadvantages of nanoparticle- mediated drug delivery to the brain (Dinda and Pattnaik, 2013; Wohlfart et al., 2012; Celia et al., 2011). As mentioned before the BBB consists of a very tight endothelial barrier, prohibiting the penetration of molecules larger than 400Da. Therefore, it is less likely that particles will be able to cross the BBB and deliver drugs to the brain. The size composition and uptake mechanisms of nanoparticles are certainly different from CNS drugs. Nanoparticles are, obviously much larger than the small drug molecules and are only able to enter the brain capillary endothelial cells via receptor or adsorptive-mediated endocytosis. Afterwards transcytosis of the nanoparticle to the basal side

of the brain endothelial cells is required for them to reach the brain parenchyma (Ragnaill et al., 2011; Zensi et al., 2009).

The success of the colloidal carriers relies on the fact that endogenous biological transport processes are generally on the scale of nanometers, and by using them, the nanoparticles have the opportunity to access the previously inaccessible disease sites of brain. Furthermore, the immune system is less effective in removing particles smaller than several hundred nanometers, allowing a long circulation before clearance. Colloidal carriers, because of their small size (in the same size range of several protein–lipid clusters usually transported by cells) and large surface area (which functions as a scaffold for plasma proteins and in this way turns nanoparticles in biological entities) are a promising method for CNS therapeutics (Ragnaill et al., 2011).

One of the distinct advantages of particulate carriers is their ability to carry larger amount of drug (higher drug load), depending on their structure. Using this approach, the uptake of a single particle into the endothelial cells would transport many drug molecules, since particles are more effective in transport than ligand-drug conjugates (one or few molecules transported). An additional benefit of the nanoparticles is that no chemical modifications of the drug molecule are required for their brain delivery, since nanoparticles can mask the inadequate properties of the drug for brain delivery, by incorporating the molecules in their matrix (Wohlfart et al., 2012). The particle size and surface characteristics of nanoparticles can be easily modified for controlled and targeted drug delivery. Depending on the application and method of preparation, different drug carriers like nanoparticle, nanospheres, nanocapsules, drug polymer conjugates, dendrimers and liposomes could be obtained (**Figure 3**).

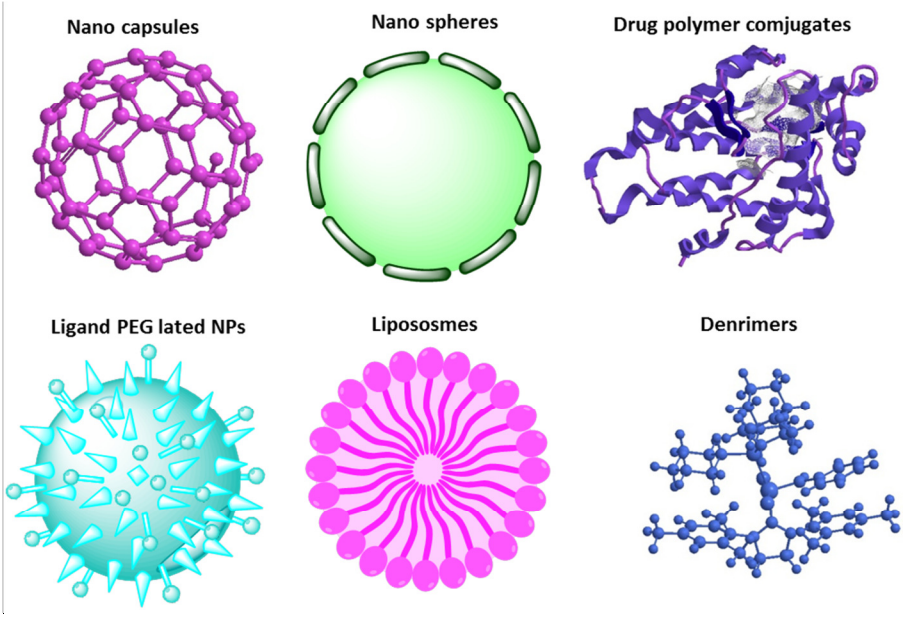

Figure 3. Different nanocarriers used for drug delivery. Nanocapsules are colloidal carrier systems in which the drug is confined to a cavity surrounded by a polymer membrane; whereas in nanospheres the drug is physically and uniformly dispersed. Liposomes are self-assembled particulate drug carrier systems, which are formed spontaneously by dispersion of phospholipids in aqueous media. Nanometre-sized hyperbranched macromolecules consisted of a central core, branching units, and terminal functional groups are called dendrimers.

In order to achieve site-specific action of the drug at therapeutically optimal rate and dose regimen, certain aspects should be considered while designing nanoparticles as a delivery system, which include, particle size, surface properties and release profile of the pharmacologically active agent (Kreuter et al., 2002). In addition, nanoparticle drug delivery systems also have the advantage of stability and long term storage, compared to other delivery methods. Moreover, nanosizing leads to increased solubility of components, reduction in the dose via improved absorption of active ingredient and higher intercellular uptake. Nanoparticles can penetrate sub-mucosal layer because the micro particles are predominantly localized on epithelial lining. Nanoparticle drug delivery can be directly administered into systemic circulation without the problem of particle aggregation or blockage of fine blood capillaries. However, the brain targeting efficiency of the nanoparticles could be considerably improved by several modifications.

1.5.8. Advancements in the nano drug delivery methods for brain

Major breakthrough in the field came when these hydrophilic polymers were used to efficiently coat conventional nanoparticle surface, to produce an opposing effect on the uptake by the mononuclear phagocytes system (Storm et al., 1995). In the past decade, a great deal of work has been devoted to develop PEGlated nanoparticles, which

are invisible to macrophages or phagocytes. Such advancement in the nanoparticle system enabled them to deliver the chemotherapeutic drug, doxorubicin to treat brain tumors, which until then was shown to have limitations in crossing BBB after intravenous administration.

Delivery of nanoparticles to the brain is based on several factors like polymer choice, surfactant and ligand attachment (Xia et al., 2011). Though, the nanoparticle as a drug carrier increased the drug delivery to the brain, it has limited capacity to cross BBB. So the surface modification or functionalization of nanoparticles became important in order for them to pass the BBB. An example of the surface modified nanoparticle is shown in **Figure 4**.

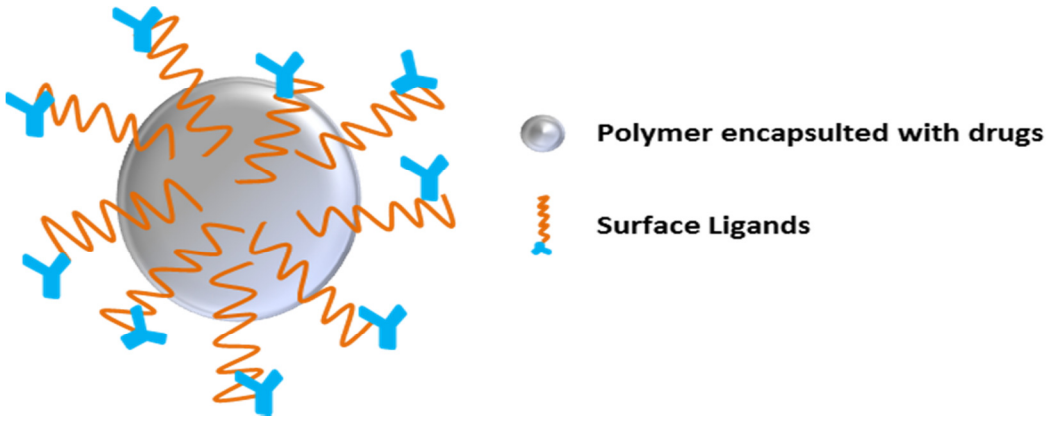

Figure 4. A model picture for Surface modified Nanoparticle. Nanoparticles surface engineered with specific ligands for site specific delivery of the drug.

In fact, the polymer, surface ligands and encapsulated therapeutic agents, all can be modified. This flexibility allows control over release kinetics, cell targeting, and treatment strategy, respectively. The kinetic process of surface modified nanoparticles and conventional therapies are expressed in **Figure 5**.

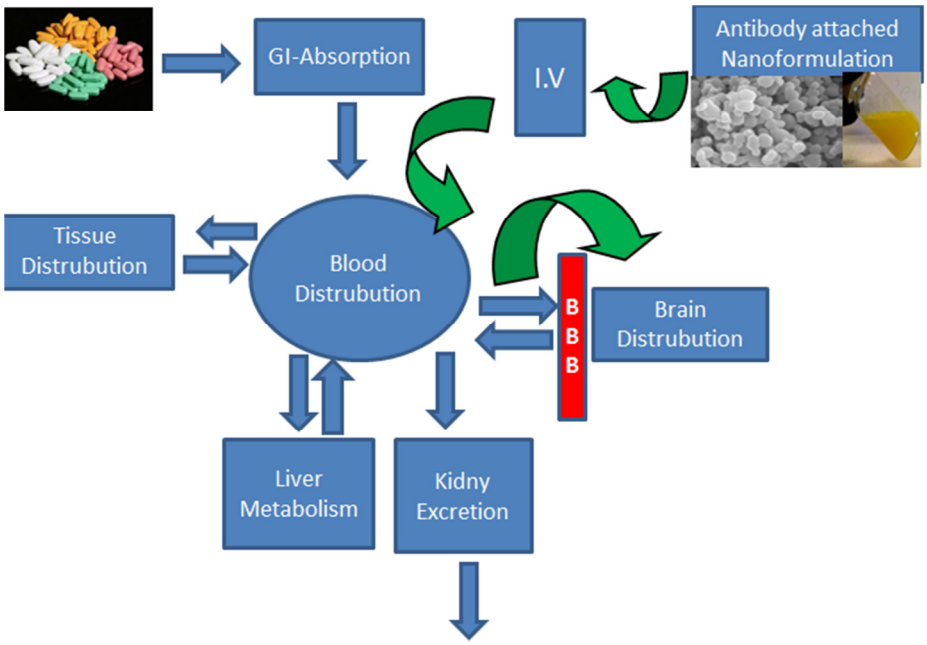

Figure 5. Scheme comparing the kinetics of a conventional drug taken orally and the functionalized herbal nanoformulation administered intravenously, for brain delivery. The orally taken drug first undergoes gastro intestinal (GI) absorption followed by distribution, metabolism and excretion, greatly reducing the dosage of drug. Since the nanoformulation is modified to cross the first pass metabolism and functionalized with brain protein specific antibody in its surface to cross the BBB, they reaches the site of action (brain) very soon.

Functionalization of nanoparticles enable them to deliver the drug at a rate directed by the needs of the body, over a period of treatment (Mahon et al., 2012). Also, it channels the active entity of drug to the site of action. Thus, surface modified/functionalized nanoparticles has significant advantage of achieving the desired pharmacological response at a selected site without undesirable interactions with other sites, so that we can expect the drug to have a specific action with fewer side effects and better therapeutic index. Conventional dosage forms including prolonged-release dosage forms are unable to meet these characteristics.

1.5.9. Herbal extracts or compounds based nanoformulations

A number of plant constituents like flavanoids, tannins, terpenoids have shown an enhanced therapeutic effect when incorporated into novel drug delivery vehicles at similar or lower dose compared to conventional plant extracts. Recently, great attention has been given to the development of herbal nanoformulations (Ansari et al., 2012). Curcumin is being used for centuries as remedy for many diseases including neoplastic, neurological, cardiovascular and pulmonary disorders (Duan et al., 2010). Despite the favorable properties of curcumin, its features such as, extremely low solubility in water, poor bioavailability and degradation at alkaline pH, have greatly

limited its *in vivo* applications. Curcumin loaded nanoformulations could deliver curcumin into tissue macrophages, specifically bone marrow and splenic macrophages in rats and suggested that the intravenous delivery system of curcumin using lipid-based nanoparticles may be best for antioxidant and antiinflammatory therapies (Sou et al., 2008). The cellular uptake of curcumin could be greatly enhanced by encapsulating curcumin into nanoparticles (Liu et al., 2012).

Cuscuta chinensis is a commonly used traditional Chinese medicine to nourish liver and kidney (Yen et al., 2008). Due to the poor water solubility of its major constituents such as flavonoids and lignans, its absorption upon oral administration is limited. Oral administration of *C. chinensis* extract as nanoparticles exhibited 5 times higher hepatoprotective and antioxidant effects compared to equivalent concentration of *C. chinensis* ethanolic extract. In addition, the results demonstrated that hepatoprotective and antioxidant effects of 50 mg/kg *C. chinensis* nanoformulation are more effective than the ethanolic extract at a dose of 125 mg/kg. Consequently, it has been suggested that absorbency and solubility of poorly soluble herbal medicines could be improved by employing nanoparticles (Yen et al., 2008). Similarly, nanoformulation was also developed for quercetin, a well-known flavonoid compound found in some plants, foods, and beverages, and has antioxidant, anti-inflammatory, anticancer, and antiviral properties. Quercetin is poorly soluble in water, which has limited its absorption following oral administration. After fabricating as nanocrystals, the dissolution rate and antioxidant activity were enhanced. In fact, the reducing power of the quercetin nanocrystals were more effective than the original quercetin (Sahoo et al., 2011).

Chemotherapeutic drugs such as paclitaxel, a major anticancer drug isolated from the bark of *Taxus brevifolia*, which exhibits activity against malignant glioma and brain metastases have been explored for the treatment of brain diseases using nanoparticles. When compared to the commercially available formulation, Taxol with Paclitaxel loaded nanoparticles (methoxy poly(ethylene glycol)– poly(ε-caprolactone) nanoparticles), demonstrated increased brain uptake and therapeutic index (Ajazuddin and Saraf, 2010). We summarizes the list of herbal nanoformulations, their biological activity, and the rationale for their nanoencapsulation in Table 3.

Table 3. Herbal extract and isolated compounds based nanoformulations

Active compound(s)/ Plant species	Biological activity	Formulation	Aim for Nanoencapsulation	Reference
Curcuminoids	Anticancer, Antioxidant, Neuro protection	SLN, NE, LP	To enhance bioavailability, cellular uptake, prolonged release & targeted drug delivery	(Lim et al., 2011; Anand et al., 2010)
Triptolide	Antiinflammatory	NP SLN	To enhance drug penetration into skin & to decrease toxicity	(Mei et al., 2003)
Flavonoids	Hepato protective, Antioxidant	NP	To protect orally delivered flavonoids from GI degradation	(Leonarduzzi et al., 2010)
Artemisinin	Anticancer	NP	To achieve sustained release	(Chen et al., 2009)
Beriberine	Anticancer	NP	To achieve sustained release	(Khemani et al., 2012)
Camptothecin	Anticancer	NP	For site specific delivery	(McCarron et al., 2008)
Sylibin	Anti ulcersctive colitis Hepatoprotective, Anticancer	SLN	Ulcer active Colitis, To obtain Anti-diabetic activity & to treat oral carcinoma	(Varshosaz et al., 2015; Das et al., 2014; Gohulkumar et al., 2014)
Paclitaxel	Anticancer	NP	To improve therapeutic index	(Danhier et al., 2009)
Docetaxel	Anticancer	NP	To improve bioavailability& cellular uptake	(Musumeci et al., 2006)
Resveratrol	Antioxidant, Anticancer	SLN	To enhance bioavailability	(Teskac and Kristl, 2010)
Cuscuta chinensis	Hepatoprotective	NP	To increased water solubility	(Yen et al., 2008)
Cassia auriculata	Antidiabetic	Gold-NP	To increase activity & stability	(Kumar et al., 2011)
Zingiber officinale	Antioxidant	Gold-NP	To achieve anti-bacterial activity	(Velmurugan et al., 2014)
Quercetin	Antioxidant Antiinflammatory	SLN	To improve brain delivery & to enhance dissolution rate	(Dhawan et al., 2011)

NP-Nanoparticles**, SLN-** Solid lipid nanoparticles, **NE**- Nano emulsion**, LP**-Liposomes

1.5.10. Herbal nanoformulations for delivery to the brain

The potential therapeutic effect of herbal medicine is becoming more popular in brain drug delivery (Kennedy and Wightman, 2011). Though herbal medicines are safe and are less toxic, to achieve better effect in the brain some modification is needed in the delivery methods. A limited numbers of work have been published on herbal nanoformulation for the brain delivery, which are summarized in **Table 4**.

Table 4. Nano herbal formulation specific for brain delivery

Plant/ Active compound	Biological activity	Reasons for Nanoencapsulation	References
Ginkgo biloba	Neuroprotection Brain activation	To improve the cerebral blood flow & metabolism	(Han et al., 2012)
Quercetin	Antioxidant	To improve brain delivery, Increase anti-oxidant activity	(Kumari et al., 2011)
Hypericum species	Antioxidant Neuroprotection	Protect against oxidative damage	(Prakash et al., 2010; Zeisser-Labouebe et al., 2006)
Piperine	Antiinflammatory Antioxidant Antidepressant	To decrease toxicity & to increase water solubility	(Chonpathompikunlert et al., 2011)
Curcumin	Neuroprotection Antioxidant	To increase the retention time and bioavailability	(Kundu et al., 2012)

On of the most important compounds exploited for potent antioxidant and anti-inflammatory activities is curcumin. Curcumin could chelate the redox active metals iron and copper (Baum and Ng, 2004). It is considered extremely safe even at very high doses. However, it has restrictive pharmaceutical role because of its very low aqueous solubility, rapid systemic elimination, inadequate tissue absorption, poor absorption from the gut and degradation at alkaline pH, which severely curtails its bioavailability (Anand et al., 2007;Wahlstrom and Blennow, 1978). For instance, curcumin dose of 0.1 g/kg via intraperitoneal route showed that only a trace amount (0.4 μg/g) in brain tissue (Pan et al., 1999). However, when administered as curcumin loaded PLGA nanoparticles, it exhibited the capability to cross BBB and significantly prolong the retention time of curcumin in the cerebral cortex (increased by 96%) and hippocampus by 83% (Tsai et al., 2011). In another study curcumin-loaded lipid-core nanocapsules were developed to achieve improved antiglioma activity. In C6 and U251MG gliomas, these curcumin-loaded lipid-core nanocapsules promoted a biphasic delivery of curcumin with enhanced cytotoxicity compared to free (non-encapsulated) curcumin in mice. In rats bearing C6 gliomas, curcumin-loaded lipid-core nanocapsules (1.5 mg/kg/day, i.p.) decreased the tumor size, malignance and prolonged animal survival, compared to the same dose of non-encapsulated free curcumin. In addition, curcumin-loaded lipid-core nanocapsules administration did not alter serum markers and histological parameters of tissue toxicity. Collectively, the data suggested that the nanoencapsulation of curcumin in lipid-core nanocapsules is a promising strategy to improve its pharmacological

efficacy in the treatment of gliomas (Zanotto-Filho et al., 2013). Curcumin nanoparticles have also been used to treat Alzheimer's disease (AD). Curcumin-conjugated nanoliposomes, with curcumin exposed at the surface, were designed to treat AD. They appeared to be monodispersed, stable and non-toxic in vitro. They down regulated the secretion of amyloid peptide and partially prevented Aβ-induced toxicity. They strongly labeled Aβ deposits in post-mortem brain tissue of AD patients and APPxPS1 mice. Injection in the hippocampus and in the neocortex of these mice showed that curcumin conjugated nanoliposomes was able to specifically stain the Aβ deposits *in vivo*. Curcumin-conjugated nanoliposomes could find application in the diagnosis and targeted drug delivery in AD (Lazar et al., 2013**).** Other herbal medicines have also been developed as nanoformulations. Extract of *H. perforatum* was encapsulated into gold nanoparticles (Nanohypericum-HPGNPs) and used for its protective role against restraint stress-induced behavioral and biochemical alterations in mice. The study report proved that Nanohypericum-HPGNPs had good neuroprotective activity compared to *H. perforatum* extract (Prakash et al., 2010). Quercetin is another natural flavonoid compound, which is reported to possess anticancer, antifree radical, antianaemic, antiinflammatory, and antiallergic properties. Nanoencapsulation of quercetin improved its bioavailability and absorption (Pool et al., 2012).

1.6 Nanotechnological approaches for anti-microbial activities

In addition to its application in various fields of science and industry, nanotechnology is becoming more popular in the area of microbiology in recent years. The natural antibacterial metals, such as silver, zinc, copper and gold possess showed better antibacterial activity, when converted as a nano sized materials. The small size and high surface to volume ratio enables the metallic nanoparticles to intimately interact with the microbial membrane. In general the metal nanoparticles like silver and gold are synthesized by variety of chemical and physical methods, which required organic solvents and toxic chemical. The development of plant based green approaches for preparing silver nanoparticles is evolving as an important branch of nanotechnology in recent years. Preparing nano size particles using plant based natural sources without the use of any harmful chemicals or solvents is called green synthesis. Green synthesized silver nanoparticles have shown greater anti-bacterial (Marslin et al., 2015) and antifungal (Kathiravan et al.,2015) activities.

1.7 Objectives

In the present endeavor, we focused on two important aspects of nanotechnology application regarding plants such as, encapsulation of plant bioactive compound or crude extract in polymeric nanoparticles to enhance their neuroprotective activity and to green synthesize silver nanoparticles using plant extract to promote antimicrobial activity. The present work was undertaken with the following specific objectives:

1. To develop a simple, fast and reproducible HPLC-RP method for the identification of withanolides from *W. somnifera* and curcumin from *Curcuma* species.
2. To prepare and characterize PCL and MPEG-PCL nanoparticles loaded with *W. somnifera* extract and curumin
3. To study the cellular uptake of *W. somnifera* extract and curumin loaded PCL and MPEG-PCL nanoparticles *in vitro*
4. To evaluate the protective effect of *W. somnifera* extract and curumin loaded PCL and MPEG-PCL nanoparticles against tBHP induced oxidative damage in U251 glioma cells
5. To visualize the biodistribution of *W. somnifera* extract and curumin loaded PCL and MPEG-PCL nanoparticles in an *in vivo* model
6. To green synthesize silver nanoparticles using *W. somnifera* extract, their characterization and evaluation of antibacterial activity in cream formulation.

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Chapter 2:
Preparation and characterization of extracts from *Withania somnifera* and *Curcuma sps*

2. Preparation and characterization of extracts from *Withania somnifera* and *Curcuma* sps

2.1 Abstract

W. somnifera, *C. longa* and *C. aromatica* methanolic extracts were prepared and characterized by high performance liquid chromatography (HPLC) analysis. Different *W. somnifera* plant parts (leaf, root, fruits) and rhizomes (turmeric) of *Curcuma* species were tested. There is no method available for the quantification of all known withanolides (withanolide-A, withanolide-B, withanone, withaferin-A, and 12-deoxy-withastramonolide) simultaneously. By studying the influence of column matrix, eluent composition and mobile phase in the resolution of compounds, we have developed a reverse phase HPLC (RP-HPLC) method. Among the different columns tested, Purospher C-18 and Lichrospher C-18 exhibited the best resolution of *W. somnifera* compounds. On the other hand, Purospher C-18 column was used to analyze extracts of *C. longa* and *C. aromatica* rhizomes to quantify curcumin.

2.1. Introduction

W. somnifera is referred as Indian ginseng due to its high medicinal value and nontoxic property. Various parts of this plant have been used to treat various neurological disorders, geriatric debilities, arthritis, stress and behaviour-related problems (Dhuley, 2001;Pratte et al., 2014). Currently, different part (leafs, roots, fruits) of this plant are used all over the world as decoction, infusions, ointment powder and syrup. Moreover, raw plant material and extracts of this species are sold as dietary supplements in the USA for increasing energy and endurance, strengthening immune function, and helping the body overcome imbalance (Alam et al., 2011). Although a few HPLC and TLC methods are available for the quantification of withaferin A (Chib et al., 2013; Ganzera et al., 2003; Khajuria et al., 2004; Murthy et al., 2008; Mundkinajeddu et al., 2014), there is no adequate method for the separation and quantification of all known withanolides (withanolide-A, withanolide-B, withanone, withaferin-A, 12-deoxy-withastramonolide) simultaneously. In fact, most of these methods are disadvantages, as the acetylation of withaferin-A is required prior to analysis and the separation time is too long.

Similarly, *Curcuma* species are used for medicinal and cosmetic purposes since ancient time in India (Aggarwal et al., 2013; Gupta et al., 2013). Many pharmacological studies have revealed that the poly phenolic compound curcumin as the active compound of *Curcuma* species. For example, curcumin exhibits anti-oxidant (Ak and Gulcin, 2008), anti- cancer (Aggarwal et al., 2003), anti-inflammatory (Menon and Sudheer, 2007), anti-microbial (De et al., 2009) and extensive neuroprotective properties (Kuo et al., 2011; Ray et al., 2011). Although several analytical methods have been developed for the identification and quantification of curcumin, only limited studies are available for the quantification of curcumin from different *Curcuma* species. Hence, the main focus of the study is (i) to develop HPLC analytical method, which includes simultaneous identification and quantification of major withanolides in *W. somnifera* extracts and (ii) to compare curcumin contents of different *Curcuma* species.

2.2. Materials and methods

2.2.1. Chemical and reagents

Withanolides (withanolide-A, withanolide-B, withanone, withaferin-A, 12-deoxy-withastramonolide) standards were obtained as gift from Natural Remedies (Bangalore India). Curcumin standard was purchased from Sigma Aldrich (Germany). All the solvents used in this study were of HPLC analytical grade purchased from Merck (Germany).

2.2.2. Plant materials

W. somnifera leaf, fruit and root were collected from Bangalore, India. Rhizomes of *C. longa* and *C. aromatica* were collected from Tamilnadu, India. The collected materials were dried under shade, finely powdered and stored in an airtight container.

2.2.3. Preparation of extract

Powdered leaves, roots and fruits (*W. somnifera)* and rhizomes (*C. longa* and *C. aromatic*) were separately extracted. Briefly 200 mg sample was taken in a 20 ml glass vial and 10 ml of 90% methanol was added. Samples were subjected to sonication for 20 min under dark at ambient temperature. After sonication, 1 ml of extract was taken in an eppendorf tube and centrifuged at 5000 rpm for 5 min. Supernatant was filtered through a 0.45 µm Nylaflo membrane filter (Pall Corporation, USA) and submitted to HPLC–DAD analysis.

2.2.4. Preparation of standards

Stock solutions (500 µg/ml) of withanolides (withanolide-A, withanolide-B, withanone, withaferin-A, and 12-deoxy-withastramonolide) were prepared by dissolving these compounds in methanol and DMSO (1:1) mixture. Curcumin stock (500 µg/ml) was prepared in methanol. All the stocks were prepared in 4 ml glass vials and stored at 4 °C. For HPLC working standard, 100 µl of each withanolide stock solution (withanolide-A, withanolide-B, withanone, withaferin-A, and 12-deoxy-withastramonolide) was taken into a HPLC vial and the volume was made up to 1 ml using methanol to obtain a final concentration of 50 µg/ml of each compounds. Similarly, working standard of curcumin (50 µg/ml) was prepared methanol.

2.2.5. HPLC analysis

HPLC analyses were performed in a Merck-Hitachi LaChrom Elite high pressure liquid chromatography apparatus (Hitachi, Japan), equipped with an L-2450 DAD detector, L-2300 column oven, L-2130 quaternary pump and L-2200 auto sampler. EZChrom Elite software program was used for the data acquisition. We have tested the separation of compounds in four different columns namely Hypersil BDS 100×4cm 3 µm, Hypersil 120-5 ODS 5µm, Lichrospher RP-18 5µm and Purospher RP-18 5 µm column (Merck, Germany). Once the column was selected, separation of compounds was first tested by two-phase mobile (eluent) system. The tested compositions for two phase mobile system were (i) water/ acetic acid (0.1%) and methanol, (ii) water/formic acid (0.1%) and methanol, (iii) water/formic acid (0.1%) and acetonitrile and (iv) water/acetic acid (0.1%) and acetonitrile. To further improve the separation, we tested three-phase mobile phase (eluent) system which consist of 0.1% acetic acid- water as eluent A, 0.1% acetic acid-acetonitrile as eluent B and 0.1% acetic acid- methanol as eluent C. Detection was achieved with DAD detector and the data for all peaks were accumulating in the range 200-400 nm and the chromatograms were recorded at 235 nm.

For *Curcuma* extracts, chromatographic separation was carried out using Lichrospher RP-18 5 µm (Merck, Germany) column using water/formic acid (99:1) and methanol as mobile phases.

2.2.6. Identification and quantification of compounds

The withanolides were identified with the help UV-spectrum of standard withanolides at 235 nm. Quantification was carried out by external standard method comparing the chromatogram, retention time and area of peak of standard and extracts. In the same way curcumin was identified with the help UV-spectrum of standard at 425 nm.

2.3. Results and discussion

2.3.1 Influence of columns on the separation of withanolides

To optimize the column for identification and quantification withanolides, we have tested four types of reverse-phase-HPLC columns. Among these, Purospher RP-18 5µm column was identified as best column by the better separation (Table 1). The other columns (Hypersil BDS 100×4cm 3 µm, Hypersil 120-5 ODS, Lichrospher RP-18 5 µm) showed a weak separation and most of the time the compounds were superimposed together. The

influence of columns on the separation of withanolides from different parts of *W. somnifera* is shown below **(Figure 1-3).**

Table 1. Retention time of withanolides in Purospher RP-18 e 5µm column

Eluent Composition	Compounds Retention time	STD	Leaf	Root	Fruits
Formicacid:methanol	a	17.10	Yes		
	b	17.63	No		
	c	17.79	Yes		
	d	18.03	Yes		
	e	20.12	No		
Formicacid:actonitrile	a	8.91	Yes		
	b	11.10	No		
	c	12.73	No		
	d	12.94	Yes		
	e	17.99	No		
Aceticacid:acetonitrile	a	8.87	Yes		
	b	11.01	No		
	c	12.66	No		
	d	12.88	Yes		
	e	17.90	No		
Aceticacid:methanol	a	19.08	Yes		
	b	19.67	No		
	c	20.10	No		
	d	20.48	Yes		
	e	22.42	No		

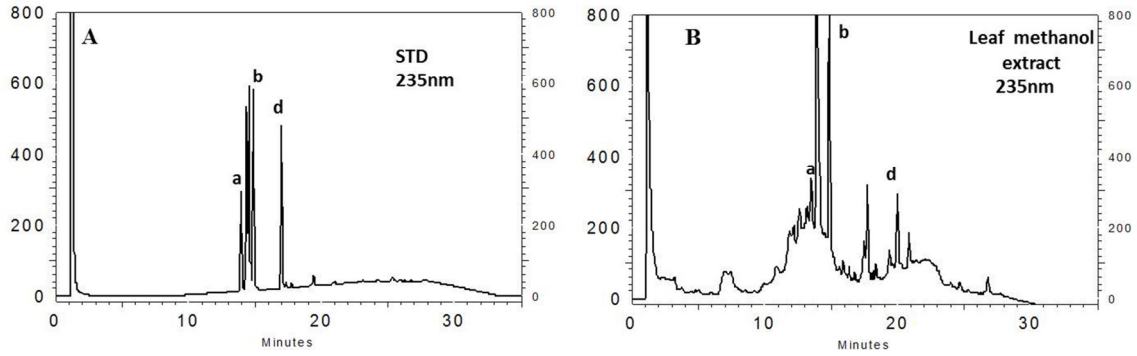

Figure 1. HPLC chromatogram recorded at 235 nm of standards (A) and *W. somnifera* leaf extract (B) showing peaks of withanolides (a- withanolide-A. b- withanolide-B, c- withanone, d- withaferin-A, e- 12-deoxy-withastramonolide) separated by Hypersil BDS 100×4cm 3 µm.

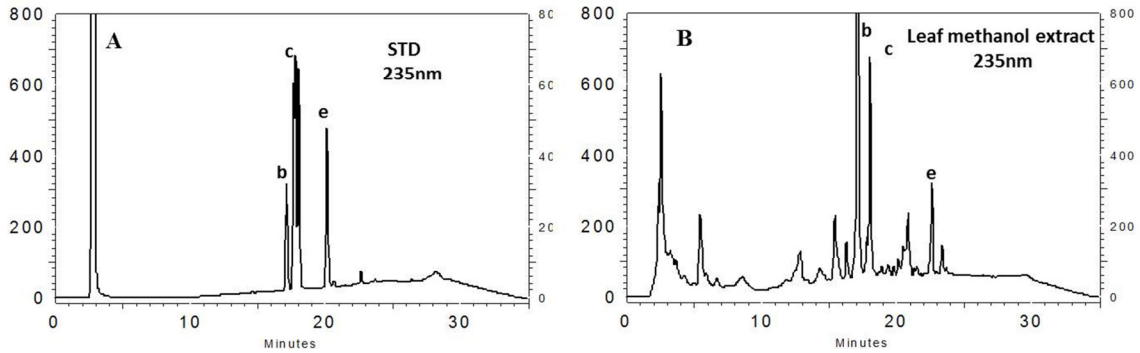

Figure 2. HPLC chromatogram recorded at 235 nm of standards (A) and *W. somnifera* leaf methanolic extract (B) showing peaks of withanolides (a- withanolide-A. b- withanolide-B, c- withanone, d- withaferin-A, e- 12-deoxy-withastramonolide) separated by Hypersil 120-5 ODS 5µm column.

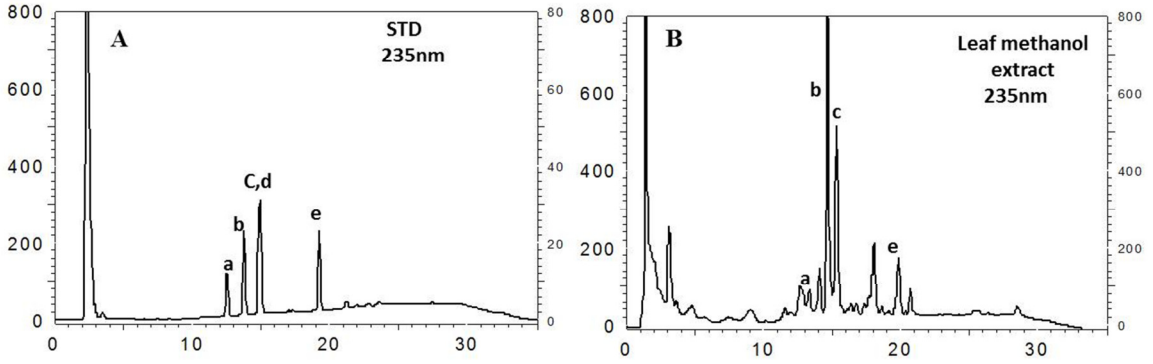

Figure 3. HPLC chromatogram recorded at 235 nm of standards (A) and *W. somnifera* leaf methanolic extract (B) showing peaks of withanolides (a- withanolide-A. b- withanolide-B, c- withanone, d- withaferin-A, e- 12-deoxy-withastramonolide) separated by Purospher RP-18 5 µm column using water/ acetic acid (0.1%) and methanol as eluent system.

2.3.2. Influence of eluent composition on the separation of withanolides

Once the optimum column was selected, to further improve the separation of withanolides, we tested different two-phase eluent system. Comparatively, a better separation was achieved in water/ acetic acid (01%) and methanol eluent system. However, a minor difficulty was noticed in the separation of withanone and withaferin-A **(Figure 3A)**. Both of these compounds (peaks c and d) were superimposed in the chromatogram posing problems in their identification and quantification. Hence, we went further to test a three-phase eluent system for HPLC identification and quantification all the withanolides. The final eluent gradient used for sample analysis is shown in **(Table 2)**. The three-phase eluent system avoided the problem related to the separation with withanone and withaferin-A observed in the two-phase eluent system. Moreover, it showed a better separation of all the five

withanolide standards (**Table 3; Figure 4A)**. However, withanolide-B could not be detected in any of the *W. somnifera* extracts (**Table 3; Figure 4)**. On the other hand, withanolide-B could be efficiently detected in the two-phase eluent system (**Figure 1-3**).

Table 2. Elution gradient selected for the analysis of the methanol extract of *W. somnifera*

Time (min)	%A	%B	%C
0	45	15	40
5	45	15	40
20	15	15	70
25	15	15	70
30	45	15	40
40	45	15	40

Table 3. Retention time of withanolides in Purospher RP-18 e 5µm column in three phase eluent system

Eluent Composition	Compounds Retention time	STD	Leaf	Root	Fruits
	A	13.56	Yes	Yes	Yes
Acetic acid:	B	15.10	No	No	No
Acetonitrile:	C	16.17	No	Yes	Yes
Methanol	D	16.67	Yes	Yes	No
	E	20.92	Yes	Yes	No

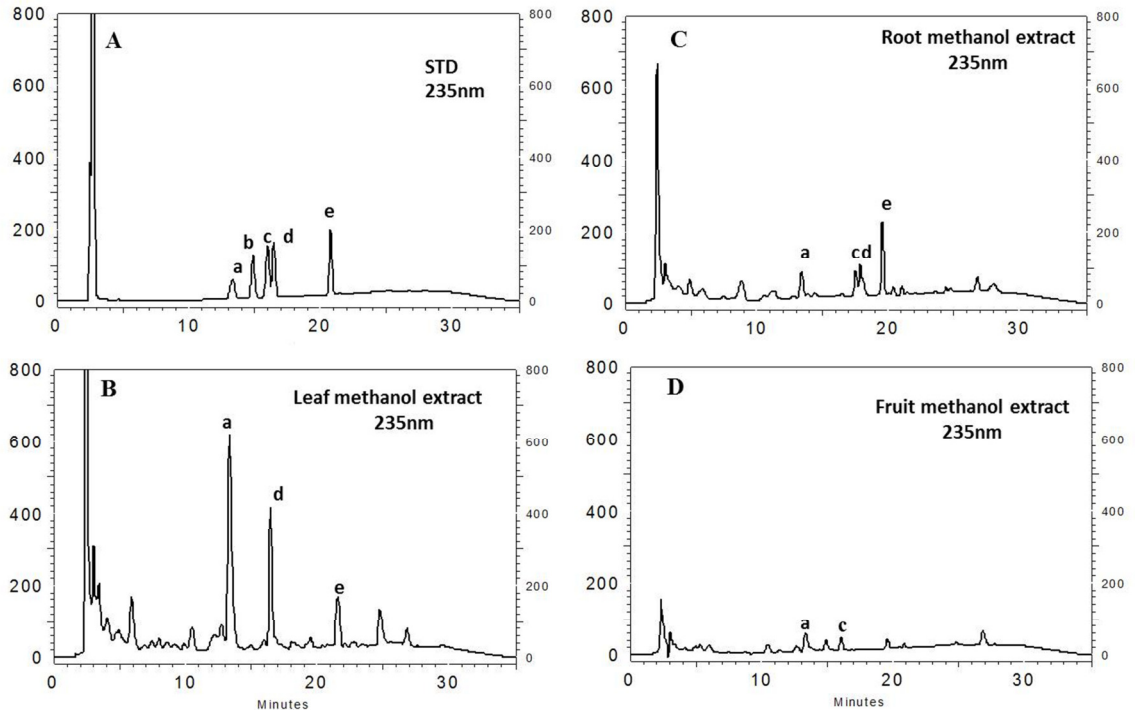

Figure 4. HPLC chromatogram of standards (A), *W. somnifera* methanolic extract of leaf (B), root (C) and fruit (D) showing peaks of withanolides (a- withanolide-A. b- withanolide-B, c- withanone, d- withaferin-A, e- 12-deoxy-withastramonolide) separated by Purospher RP-18 5 µm column.

2.3.3. Identification and quantification of withanolides

Withanolides were identified by retention time and UV spectrum. The chemical structure and UV-Vis spectrum of five standard withanolides detected Diode array are shown in **Figure 5 A-B**. From the chromatograms recorded (**Figure 4)**, we could quantify the amount of various withanolides present in different extracts by comparing the peak area of standard. However, previously reported HPLC methods were restricted to the identification of withanolide -A and withanolide-D (Ganzera et al., 2003; Bala et al., 2004). Although Khajuria and collaborators (Khajuria et al 2004) have reported simultaneous determination of four withanolides, withanolide-B could not be determined in their study. In our method, all the five standard withanolides including withanolide-B could be efficiently determined arguing for the robustness of the developed method.

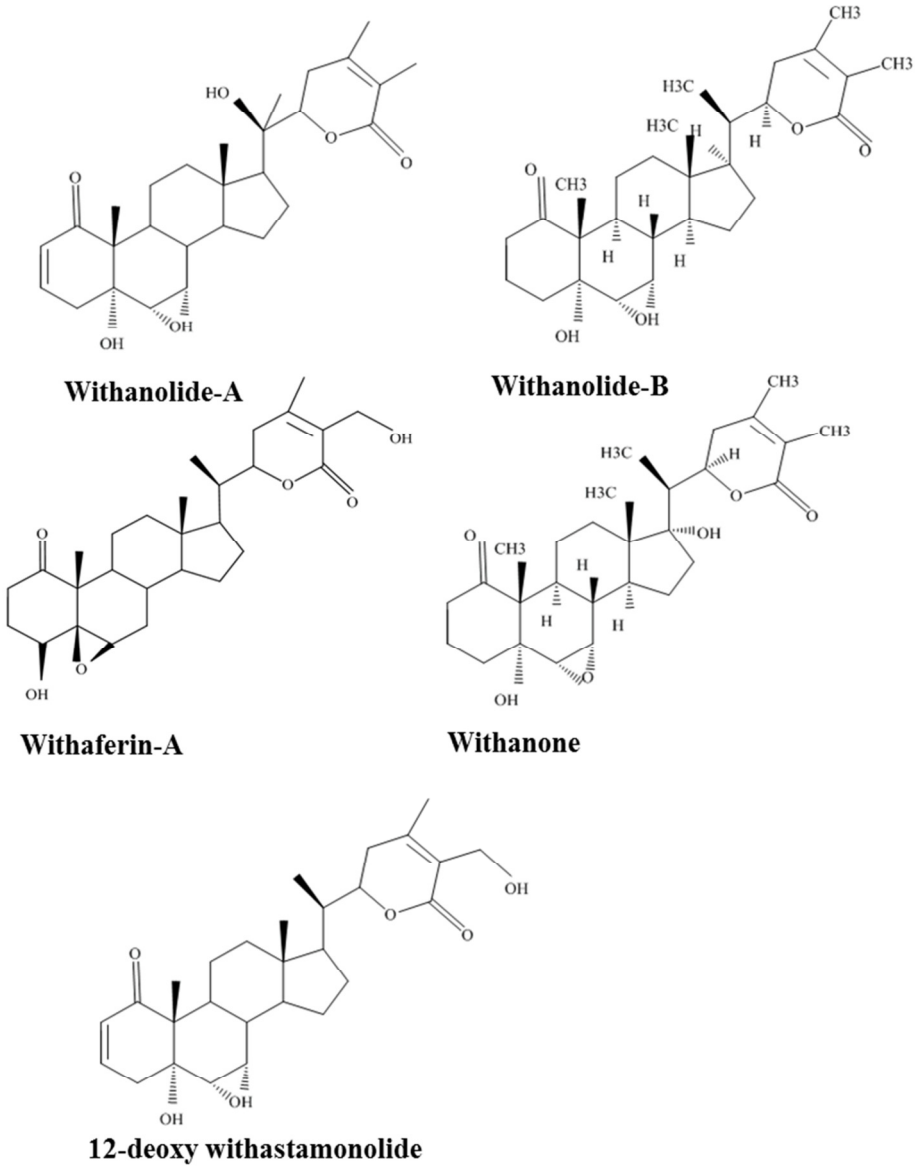

Figure 5A. Chemical structure of withanolides

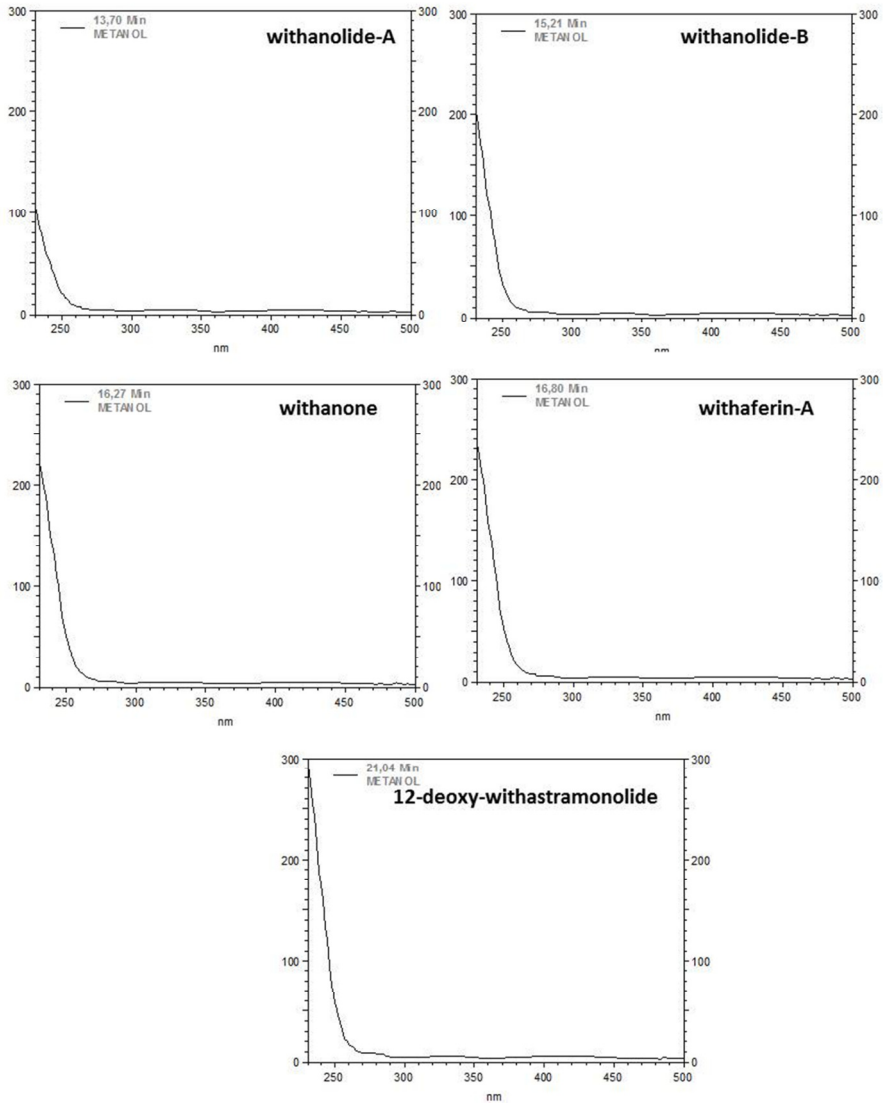

Figure 5B. UV-Vis spectrum of withanolides

Although the highest content of withanolides was present in leaf extract, we could only detect three withanolides namely withanolide-A, withaferin-A, and 12-deoxy-withastramonolide (**Table 4)**. Compared to roots, fruits contained more withanolides. The maximum number of 4 withanolides with the exception of withanolide-B could be detected in the root extract.

Table 4. Withanolides content of different parts of *W. somnifera*

Compounds	STD (µg/ml)	(mg/ g wt dry biomass)		
		Root	Leaf	Fruit
withanolide-A	50	0.098	1.344	0.100
withanolide-B	50			
withanone	50	0.058		
withaferin-A	50	0.065	0.346	0.002
12-deoxy-withastramonolide	50	0.150	0.143	

2.3.4. Identification and quantification of curcumin

Curcumin is the major compound responsible for the biological activities of *Curcuma* sp. Since many HPLC methods have been reported for identification and quantification of curcumin (An et al., 2014; Wichitnithad et al., 2009), we restricted our analysis with the comparative quantification of curcumin. From the literature and the results of our thin layer chromatography (TLC) study we found that methanol is the ideal solvent for extracting curcumin. Elution gradient of curcumin in selected column for the analysis Lichrospher RP-18 5 µm is described in Table 5. Curcumin was identified by retention time and UV-Vis spectrum (**Figure 6D**). Our quantification result revealed that *C. longa* extract possessed higher amount of curcumin (1.49 mg/g dry wt biomass) than *C. aromatica* (0.55 mg/ g dry wt biomass).

Table 5. Elution gradient specification of HPLC-DAD analysis of curcumin

Time (min)	%A	%B
0	15	85
5	30	70
20	45	55
25	15	15
30	50	50
40	85	15

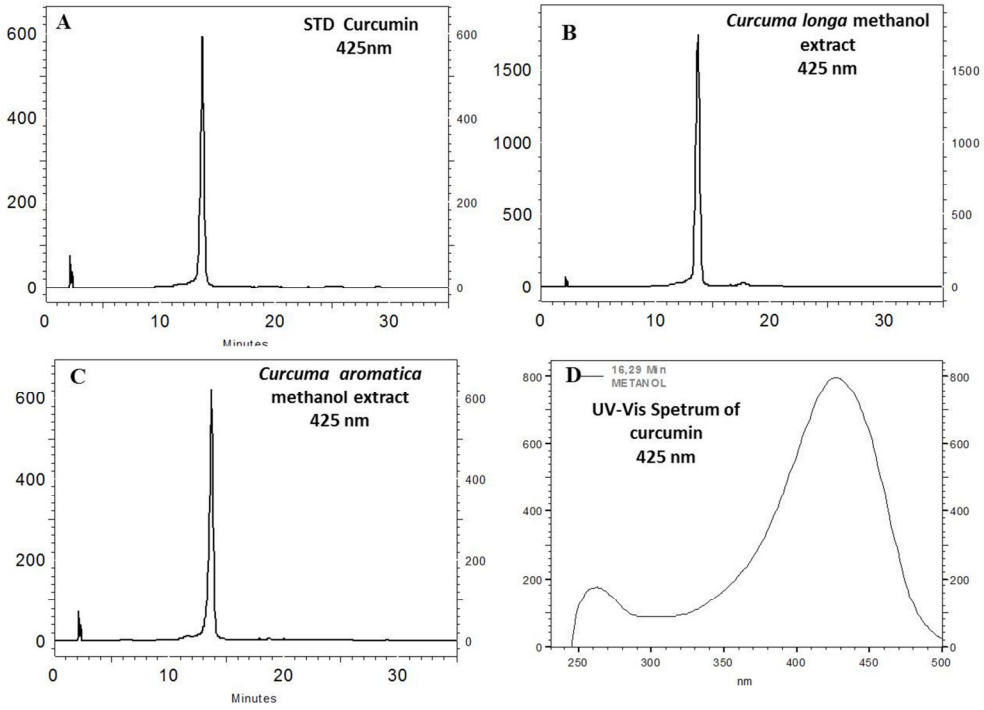

Figure 6. HPLC chromatograms of methanolic extract obtained from rhizomes of Curcuma species separated with Lichrospher RP-18 5 µm column. Standard curcumin (A), Methanolic extract of *C. longa* (B), Methanolic extract of *C. aromatica* (C). UV-Vis Spectrum of curcumin at 425 nm (D).

2.4. Conclusion

We have found that the methanolic extract obtained from leaf tissues of *W. somnifera* possessed the highest amount of withanolides compared to roots and fruits. Purospher RP-18 e 5 µm column is ideal column for the efficient separation and simultaneous quantification of withanolides. *C. longa* had higher amount of curcumin compared to *C. aromatica*.

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Chapter 3:
Preparation and characterization of *Withania somnifera* extract and curcumin loaded polymeric nanoparticles

3. Preparation and characterization of *Withania somnifera* extract and curcumin loaded polymeric

3.1. Abstract

W. somnifera extract and curcumin were encapsulated in poly -ε-caprolactone (PCL) and methoxy poly-ethylene glycol poly -ε-caprolactone (MPEG-PCL) nanoparticles by solvent displacement method. Prepared nanoparticles were characterized for their physico-chemical properties. Laser Doppler anemometry analysis revealed that the PCL nanoparticles loaded with *W. somnifera* extract ranged between 220-250 nm, whereas *W. somnifera* extract loaded MPEG-PCL nanoparticles are 50-80 nm in size. The size of curcumin loaded PCL and MPEG-PCL nanoparticles ranged between 200-240 nm and 30-70 nm respectively. TEM images revealed that all nanoparticles were spherical in shape. Analysis of WSE and curcumin content of nanoparticles synthesized using various drug-polymer ratio revealed that 1:10 ratio is the best for encapsulation. The entrapment efficacy of WSE was higher in MPEG-PCL (54.4%) compared to PCL (31.2%). Likewise, encapsulation of curcumin was higher in MPEG-PCL nanoparticles (85.8%) compared to PCL nanoparticles (72.2%). *W. somnifera* extract and curcumin loaded PCL and MPEG-PCL nanoparticles showed an initial burst release, followed by an extended release profile.

3.2. Introduction

Nanotechnology has gained much attention in the recent years due to its wide range of application in physics, chemistry, biology and medicine. Particularly, nanoparticles mediated drug carrier system has emerged as a powerful methodology due to the unique accumulation behavior of nanoparticles at the targeted site (Dumont et al., 2002). Nanoparticles are submicron-size carrier systems composed of natural or synthetic polymers with the size range of 10–1000 nm. For nanoparticle mediated drug delivery, the therapeutic agent of interest may be dissolved, entrapped or encapsulated within the biodegradable polymer matrix to achieve extended drug release over a period of time, as the polymer degrades (Marslin et al., 2009). Moreover, nanoencapsulation increases the efficacy of the drug, improves its circulation time and stability while decreases its toxic side effects (Sheeba et al., 2014).

Although, previous pharmacological studies have explored the importance of plant active compounds such as curcumin and withanolides, their clinical application is limited due to their poor aqueous solubility which decreases their bioavailability and in-vivo pharmacokinetics, compromising the efficacy (Jagetia and Aggarwal, 2007). Aqueous solubility is an important parameter for the initial phase of drug development. Approximately, more than half of the new reactive molecular entities were not successfully developed because of their hydrophobicity (Gong et al., 2010). So, it is crucial to develop water-based formulations with controlled release property in order to improve the clinical applications of plant bioactive compounds (Li et al., 2005).

Polymeric nanoparticles encapsulated with plant active compounds have been proven to be successful in increasing the bioavailability and retention time of herbal medicines. For example the neuroprotective effect of curcumin has increased after converting it into polymeric nanoformulation (Ray et al., 2011). Likewise, Dhawan et al demonstrated that solid lipid nanoparticles of quercetin, deliver the drug to the brain and have greater potential to treat Alzheimer´s disease (Dhawan et al., 2011).

Today, several polymers are in use. Among them, MPEG-PCL is an amphiphilic block copolymer composed of hydrophilic and hydrophobic segments. The characteristic structure of amphiphilic copolymers enables them to self-assemble into nanoscale core shell spherical structures with the hydrophobic part as the inner core and the hydrophilic part as the outer shell (Hu

et al., 2003). While it is easy for the hydrophobic core to incorporate lipophilic drugs, enabling their sustained release profile, the outer shell formed by the hydrophilic part help the nanoparticles to effectively escape the scavenging activity of the reticuloendothelial systems after systemic administration thereby leading to a long circulation time (Lu et al., 2013; Aditya et al., 2015; Shao et al., 2011). In this study, we have developed and characterized *W. somnifera* extract and curcumin in poly (ε-caprolactone) PCL and methoxy poly(ethylene glycol) Poly (ε-caprolactone) MPEG-PCL nanoparticles.

3.3. Materials and methods

3.3.1. Plant materials and chemicals

Authenticated samples of *W. somnifera* leaf materials were collected from Bangalore, India. After collection, leaves were dried under shade, powdered and stored in an airtight container. Standard compounds (withanolides withanolide-A, withanolide-B, withanone, withaferin-A, 12-deoxy-withastramonolide) were obtained as a gift from natural remedies, Bangalore India.

Curcumin was purchased from Sigma Aldrich, Germany. Poly(ethylene glycol) methyl ether (MPEG, Mn 2000, Fluka, USA), ε-caprolactone (ε-CL, Alfa Aesar, USA), stannous octoate (Sn(Oct) 2, Sigma, USA),3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium, MTT, Sigma, USA), Dulbecco's Modified Eagles Medium (DMEM, HyClone Thermo Scientific, USA) and fetal bovine serum (BioChrom KG, Berlin, Germany) were purchased. All the solvents used in this study are HPLC analytical grade and they were purchased from Merck (Germany).

3.3.2. Synthesis and characterization of block copolymer methoxy poly (ethyleneglycol)-poly(ε-caprolactone)

MPEG-PCL copolymer was prepared by ring-opening polymerization of ε-CL and MPEG in the presence of stannous octoate as catalyst. Briefly, 5 g of each ε-Caprolactone and 5 g of MPEG, and 0.1% catalyst were added into a 50 ml dried glass reactor (previously flamed and nitrogen purged several times) and sealed under reduced pressure (5 mmHg). The reaction was carried out at 140 °C for 24 hrs and then terminated by cooling in water. The reaction product was then dissolved in $CHCl_3$ and precipitated with an excess of methanol/ diethyl ether to obtain a white product. The product was dried under vacuum at 40 ° C for 48 hrs.

The synthesized copolymer was characterized by Fourier transform infrared spectroscopy (FTIR) by KBr pellet method (1 mg of polymer was mixed with 200 mg of KBr in mortar) and measured with Bomem MB104 193 spectrometer in the range of 4000–500 cm 194 –1. The ^{1}H nuclear magnetic resonance spectroscopy (1H NMR) analysis was performed using chloroform as solvent in a Brucker (Avance III, 400 MHz) spectrometer at a temperature of 25 ° C, with 1 second delay and 128 accumulation cycles.

3.3.3. Preparation of *W. somnifera* Extract

As discussed in the previous chapter *W. somnifera* leaf extract has the higher amount of withanolides. Hence, we selected *W. somnifera* leaves for the extraction of withanolides. Finely powdered *W. somnifera* leaves (50 g) were extracted in aqueous methanol solution (90%) with sonication for 1 hrs in the dark at ambient temperature. The liquid phase was filtered through 0.45 µM nylon membrane (Poll Corporation, USA) and purified with dichloromethane. HPLC–DAD analysis of *W. somnifera* extract was performed to confirm the presence of bioactive compounds (withanolide-A, withanolide-B, withanone, withaferin-A, 12-deoxy-withastramonolide) in the extract.

3.3.4. Preparation of *W. somnifera* extract and curcumin encapsulated nanoparticles

Both *W. somnifera* leaf extract and curcumin encapsulated nanoparticles were prepared by solvent displacement method. To prepare *W. somnifera* extract loaded nanoparticles, 100 mg of PCL or MPEG-PCL and 10 mg of *W. somnifera* extract were dissolved in 5 ml of dichloromethane. Curcumin loaded nanoparticles were prepared by dissolving curcumin and the respective polymers in acetone. These organic solutions were separately introduced into 50 ml of aqueous solution containing 1% of Pluronic (F-68) under continuous magnetic stirring for 4 hrs. The residual organic solvent was removed by placing the sample in rotary evaporator at 37 °C. After removal of organic solvent the nanoparticles suspension centrifuged at 20000 rpm for 15 min to pellet down the nanoparticles. The pelleted nanoparticles were freeze dried and stored 4 °C for further use. For the PCL nanoparticles after the preparation the nanoparticles suspension was centrifuged at 20000 rpm for 15 min to pellet down the nanoparticles. PCL and MPEG-PCL empty nanoparticles were prepared in the absence of *W. somnifera* extract or curcumin. Self-assembly of PCL and MPEG-PCL nanoparticles formation is illustrated in **Figure 1**. In order to obtain nanoparticles with optimal characteristics

different empty nanoformulations and nanoparticles encapsulated with *W. somnifera* extract /curcumin were prepared by altering the concentrations of polymer, and stabilizer (**Table 1**).

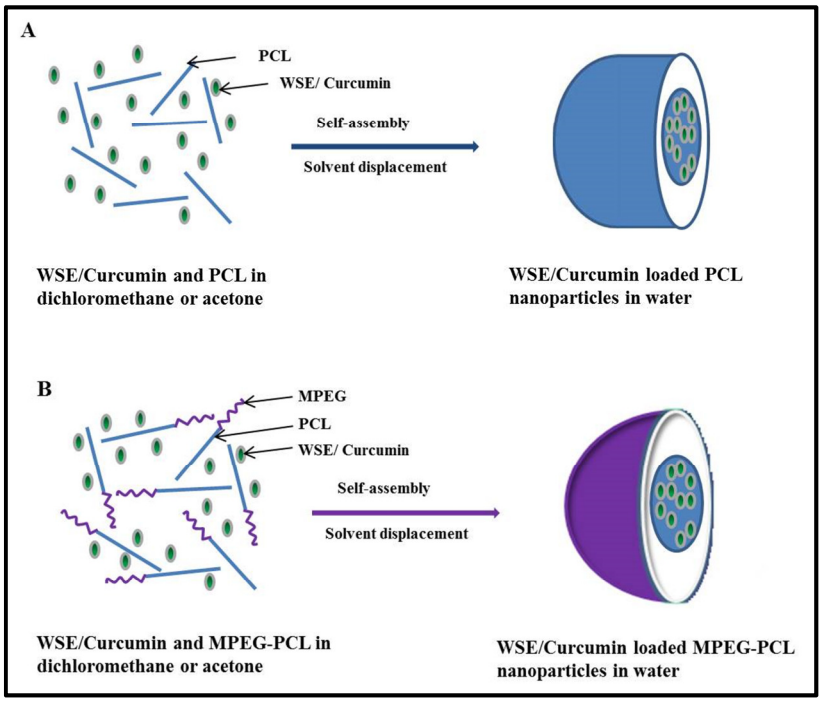

Figure 1. Schematic representation of *W. somnifera* extract (WSE) and curcumin loaded nanoparticles preparation by solvent displacement method. Preparation of PCL nanoparticles (A), Preparation of MPEG-PCL nanoparticles (B).

Table 1. Various compositions of polymer, drug and stabilizer used in the preparation of nanoparticles

Formulation code	Drug (mg)	Polymer (mg)	Organic solvent (ml)	Stabilizer (%)
PCL1	10	50	5	0.5
PCL2	10	100	5	0.5
PCL3	10	50	5	1
PCL4	**10**	**100**	**5**	**1**
PCL5	10	100	5	1.5
MPCL1	10	50	5	0.5
MPCL2	10	100	5	0.5
MPCL3	10	50	5	1

MPCL4	**10**	**100**	**5**	**1**
MPCL5	10	100	5	1.5

For *in-vitro* cellular uptake and *in-vivo* localization studies (Chapter 4), coumarin-6 co-encapsulated with *W. somnifera* extract nanoparticles were prepared following the same methods mentioned above. In brief, 100 mg of PCL or MPEG-PCL and 2 mg coumarin-6 and 8 mg *W. somnifera* extract were dissolved in 5 ml of dichloromethane. These organic solutions were separately introduced into 50 ml of aqueous solution containing 1% of Pluronic (F-68) under continuous magnetic stirring for 4 hrs and the nanoparticles were isolated as per the aforementioned method.

3.3.5. Physicochemical characterization of *W. somnifera* extract and curcumin loaded nanoparticles

3.3.5.1. Particle size, polydispersity index and Zeta potential

Particle size distribution, polydispersity index and Zeta potential of the *W. somnifera* extract and curcumin loaded PCL, and MPEG-PCL nanoparticles were determined in a Zetasizer (Zetasizer nano ZS, Malvern Instruments, UK). For the measurements, 500 µL of nanoparticle suspension was diluted with 500 µL of ultrapure water and used for the analysis. The zeta potential was determined based on the electrophoretic mobility of nanoparticles in an aqueous solution using laser Doppler velocimetry and phase analysis light scattering.

3.3.5.2. Nanoparticle morphology

Morphological characteristics of the nanoparticles were confirmed by transmission electron microscopy (TEM, JEOL-1400, 902A Jeol Ltd) after negative staining with sodium phosphotungstate solution (2%, w/w). Freshly prepared *W. somnifera* extract or curcumin encapsulated nanoparticle suspension was diluted with 500 µL of ultrapure water, dropped onto copper grid (400 mesh) and used for analysis.

3.3.5.3. X-ray diffraction (XRD) analysis

XRD studies were performed using an X-ray diffractometer (Philips, PW1710) with a horizontal goniometer. The samples were placed in the sample holder and scanned at a rate of $1^{\circ}\,min^{-1}$ from 0° to 60°.

3.3.5.4. Differential scanning calorimetry

The physical state of *W. somnifera* extract and curcumin entrapped in the nanoparticles was characterized by differential scanning calorimetry (Universal V4.7A TA Instruments). The endothermic melting temperature of the drug (*W. somnifera* extract and curcumin), polymer (PCL and MPEG-PCL) and *W. somnifera* extract or curcumin loaded PCL and MPEG-PCL nanoparticles was measured by placing 5-10 mg samples on aluminum pans with lids and scanning between 25 °C and 320 °C at the rate of 5 °C per min, under nitrogen atmosphere.

3.3.5.5. Encapsulation efficiency

Concentration of withanolides encapsulated in the nanoparticles was estimated. Briefly, 20 mg of *W. somnifera* extract loaded nanoparticles was dissolved in 2 ml of methanol subjected to sonication for 5 min to disrupt the nanoparticle structure. Then the sample was centrifuged at 10000 rpm for 10 min to get a clear supernatant for analysis. HPLC analyses were performed in a Merck-Hitachi LaChrom Elite high pressure liquid chromatography equipped an L-2450 DAD detector, L-2300 column oven, L-2130 quaternary pump and L-2200 auto sampler. Chromatographic separations were carried out at 30 °C, on a Purospher RP-18e column (Merck, Germany), using 0.1% acetic acid in water (solvent A), 0.1% acetic acid in acetonitrile (solvent B) and 0.1% acetic acid methanol as the mobile phases. *W. somnifera* extract was filtered (Nylaflo membrane 0.45 μM) prior to HPLC analysis.

As mentioned above, encapsulation efficiency of curcumin nanoparticles was also determined, but using RP-HPLC. Chromatographic separation was carried out on an RP C18 Lichrospher column (Merck, Germany), using water/formic acid (99:2) and methanol as the mobile phases and absorbance were recorded at 425 nm. The encapsulation efficiency of formulations was determined by the equation:

$$\text{Encapsulation efficiency} = \frac{\text{Amount of extract or curcumin in the nanoparticles}}{\text{Total amount used for preparation}} \times 100$$

3.3.5.6. In- vitro release profile

In- vitro release studies were carried out using pH 7.4 phosphate buffer saline (PBS) by dialysis bag method

with a molecular weight cut-off of 12,000- 14,000 Da. Twelve hours prior to usage, the semi-permeable membrane was kept in PBS solution. Briefly, 20 mg of *W. somnifera* extract loaded nanoparticles were placed in the dialysis bag. The dialysis tubes were incubated in 50 mL of pre-warmed PBS (37 °C, pH 7.4). One milliliter of the sample was withdrawn from the incubation medium and release of withanolides was quantified by HPLC as described above. After sampling, equal volume of fresh PBS was immediately added into the incubation medium.

Similarly, dialysis bag method was followed for in-vitro release studies of curcumin nanoparticles, although with small modifications. Briefly, 20 mg of curcumin nanoparticles were placed in dialysis tubes. The dialysis tubes were incubated in 50 mL of pre-warmed PBS (37 °C, pH 7.4) containing Tween80 (1% v/v). One milliliter of the sample was withdrawn from the incubation medium and release of curcumin was quantified by HPLC as described above. After sampling, equal volume of fresh PBS was immediately added into the incubation medium. The concentration of withanolides or curcumin released from the nanoparticles was expressed as a percentage of the total curcumin in the nanoparticles and plotted as a function of time.

3.3.6 Statistical analysis

All data were analyzed statistically and graphs were generated using GraphPad 5 software (Prism, USA). Data are expressed as the mean ± S.E.M., of the indicated number of experiments. The significance of the differences between the mean values was evaluated using the unpaired two-tailed Student's t-test. A difference at $p \leq 0.05$ confidence level was considered significant

3.4. Results and discussion

In the recent years, herbal medicine has gained much attention because of the therapeutic efficacy and less toxicity at even higher doses (Ribeiro et al., 2013). To enhance the therapeutic activity of herbal medicine, different strategies have been attempted. Among them, nanoparticles mediated drug carrier systems have shown a higher percentage of successes (Bharali et al., 2011). Recently, a variety of novel carriers for herbal medicines such as liposomes, nanoparticles, nanocapsules, phytosomes, nanoemulsion and microspheres have been reported with significant success rate (Ajazuddin et al., 2014). Incorporation of the herbal extracts

into these novel formulation systems have the greatest advantage to overcome the major problems, such as their bulk dosing and less absorption, enticing the attention of major pharmaceutical corporations.

In addition, drugs encapsulated into a polymer matrix also provide other valuable aspects including protection from degradation, enhanced drug solubility, targeting ability of the drug to the specific site and less toxicity (Aditya et al., 2015). In this work we have encapsulated *W. somnifera* extract and curcumin successfully in PCL or MPEG-PCL polymer by solvent evaporation method.

3.4.1. Characterization of block co-polymer

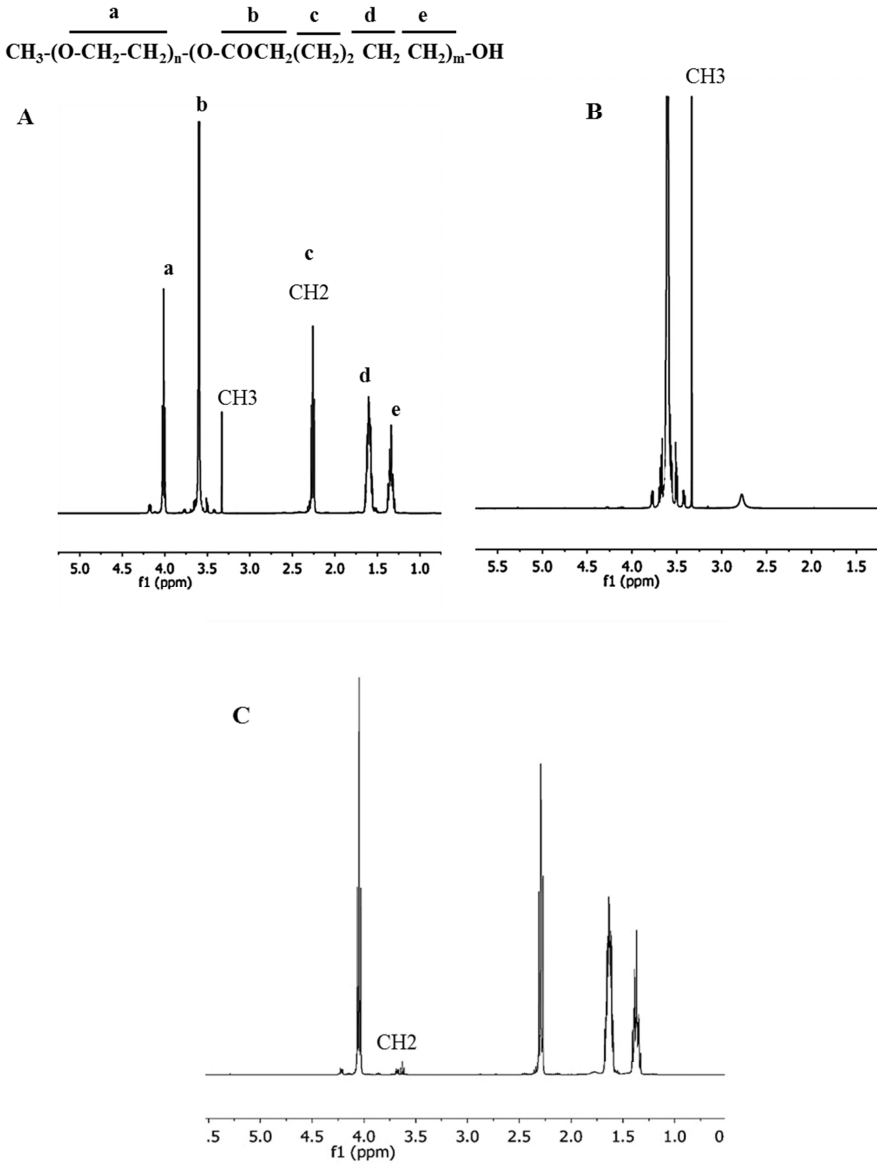

Figure 2. The ^{1}H NMR spectrum of the MPEG-PCL copolymer (A), MPEG (B), PCL (C).

MPEG-PCL block copolymers were synthesized by ring opening polymerization of ε-CL in the presence of MPEG with catalyst stannous octoate. The copolymers were characterized by ^{1}H NMR. The ^{1}H NMR spectrum of the MPEG-PCL is shown in (**Figure 2A**). The peaks at 1.30-1.33, 1.57-1.62, 2.3-2.28 and 3.33 ppm were assigned to the methylene protons in the PCL unit. The peaks at 3.5 ppm were attributed to the methylene protons of the PEG units. Presences of these peaks have confirmed the formation of MPEG-PCL copolymer.

The FT-IR spectrum for a typical MPEG-PCL copolymer is shown in (**Figure 3)**. The MPEG-PCL copolymer clearly exhibited peaks characteristic of both MPEG and PCL. The absorption band at 1727 cm^{-1} was attributed to C=O stretching vibration of ε-CL. The absorption band at 2890 cm^{-1} and 2946 cm^{-1} were attributed to C-H stretching vibration of MPEG-PCL and ε-CL, confirming the formation MPEG-PCL.

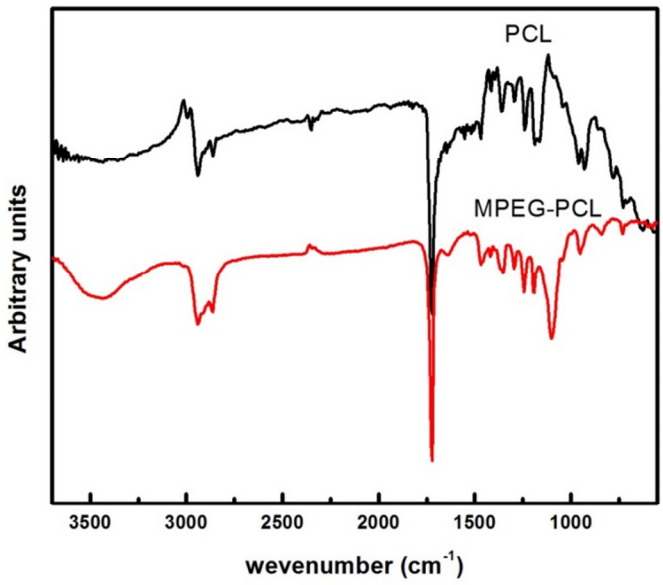

Figure 3. FT-IR spectra of PCL (black) and MPEG-PCL (red) polymer.

3.4.2. Physicochemical characterization of nanoparticles

MPEG-PCL nanoparticles have greater potential in drug delivery, it is already used to deliver several drugs with good successes rate (Peng et al., 2015; Wang et al., 2014). It is well known that the attachment of PEG in the nanoparticle enhances the circulation time and cellular up take of nanoparticles (Jokerst et al., 2011; Vij et al., 2010). Curcumin or withanolides are hydrophobic agents not soluble in water but when *W. somnifera* extract or curcumin were encapsulated into PCL or MPEG-PCL as nanoparticles, they have shown water solubility, suggesting that nanoencapsulation enhances aqueous solubility of the compounds (**Figure 4, 7**). Poorly water soluble drugs often require high doses in order to reach therapeutic plasma concentrations, after oral administration. Low aqueous solubility is the major problem encountered by the formulation of new chemical

entities as well as generic development. Any drug to be absorbed must be present in the form of an aqueous solution at the site of absorption. Water is the solvent of choice for liquid pharmaceutical formulations. Most of the drugs are either weakly acidic or weakly basic having poor aqueous solubility (Savjani et al., 2012). Since encapsulation of *W. somnifera* extract or curcumin into PCL or MPEG-PCL nanoparticles increased their water solubility, we suggest the usage of these nanoparticles may be a simple way to improve the absorption of any non-water soluble compounds.

3.4.2.1 Characterization of empty nanoparticles

The PCL and MPEG-PCL empty nanoparticles were prepared and characterized by Particle size, Polydispersity index (PDI) and Zeta potential (**Table 2**).

Table 2. Particle size, PDI and Zeta potential of Free PCL and MPEG-PCL nanoparticles

Formulation code	Zeta average size (nm ± SD)	Polydispersity index (PI ± SD)	Zeta potential (mV ± SD)
EMP1	210.20±4.24	0.21±0.24	-24.6±2.2
EMP2	226.12±4.6	0.26±0.30	-36.12±2.1
EMP3	201.38±3.2	0.32±0.24	-31.10±3.1
EMP4	**204. 32±3.3**	**0.26±0.16**	**-32.24±1.6**
EMP5	214. 3.7±3.2	0.20±0.26	-27.14±1.8
EMM1	54.30±3.60	0.32±0.220	-17.5±0.70
EMM2	70.12±2.70	0.36±0.146	-15.3±1.22
EMM3	36.44±1.62	0.34±0.006	-13.5±1.0
EMM4	**31.16±2.19**	**0.32±0.032**	**-9.3±1.40**
EMM5	48.60±2.36	0.30±0.0627	-10.7±1.32

Values are expressed as mean ± standard deviation (n=3). EMP: Empty PCL nanoparticles; EMM: Empty MPEG-PCL nanoparticles.

3.4.2.2. Characterization of W. somnifera **extract loaded nanoparticles**

Neuroprotective compounds present in *W. somnifera* extract were successfully extracted and purified with dichloromethane and successfully encapsulated as PCL and MPEG-PCL nanoparticles by solvent displacement method. Both prepared nanoparticles were completely dispersed in aqueous media without any aggregates, whereas free *W. somnifera* extract exhibits poor aqueous solubility **(Figure 4**).

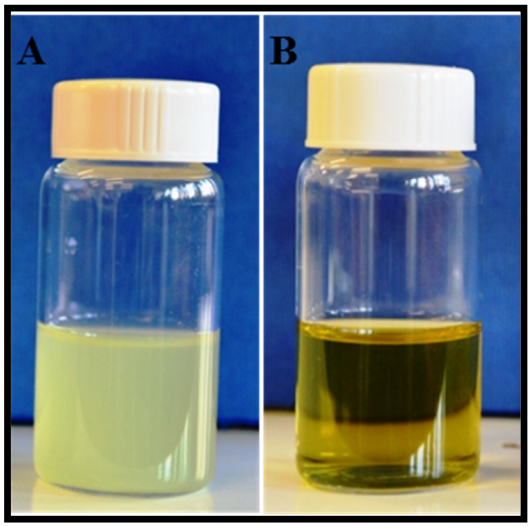

Figure 4. Aqueous solubility of *W. somnifera* extract in PCL nanoparticles (A) MPEG-PCL nanoparticles (B)

Particle size is an important parameter as it can directly affect the physical stability, cellular uptake, bio-distribution, and drug release from the nanoparticles. The prepared *W. somnifera* extract loaded nanoparticles were in the range of 214.25±2.3 to 268.32±3.2 nm for PCL nanoparticles, whereas the particles were much smaller in the rage of 30.76±1.29 to 62.32±3.7 for MPEG-PCL nanoparticles as shown in table 2. The Dynamic light scattering (DLS) characterization of size and size distribution of *W. somnifera* extract encapsulated in PCL and MPEG-PCL nanoparticles are shown in **Figure 5**. Difference in particle size was observed by altering the amount of polymer and stabilizer. The zeta potential value of MPEG-PCL nanoparticle (-7.9±1.60 mV) were much smaller compared with PCL nanoparticles (-30.14±2.6 mV) **(Table 3)**.

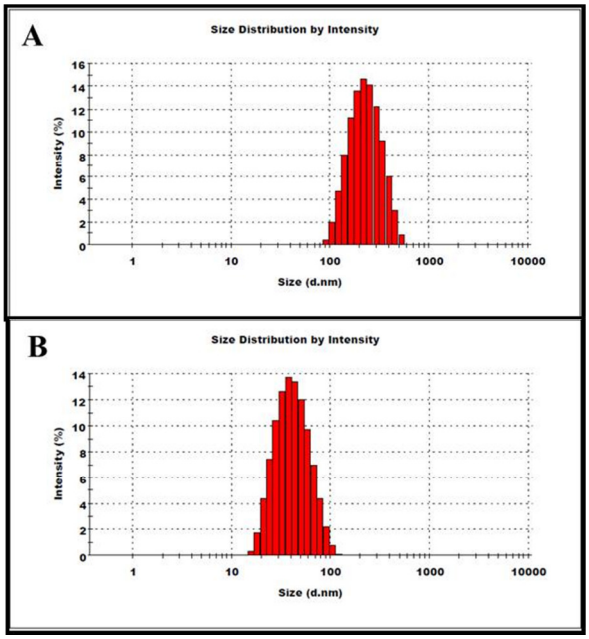

Figure 5. Particles size and distribution of *W. somnifera* extract loaded PCL nanoparticles (A), Particles size and distribution of *W. somnifera* extract loaded MPEG-PCL nanoparticles (B)

Table 3. Particle size, PDI, Zeta potential and Entrapment efficiency of *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles

Formulation code	Zeta average size (nm ± SD)	Polydispersive index (PI ± SD)	Zeta potential (mV ± SD)	Entrapment efficiency (% ± SD)
WSEP 1	242.12±3.24	0.31±0.02	-34.2±2.2	39.08 ± 2.15
WSEP 2	268.32±3.6	0.36±0.20	-32.42±3.1	46.06 ± 3.52
WSEP 3	240.28±4.2	0.28±0.14	-30. 20±2.1	42.16 ± 2.32
WSEP 4	214. 25±2.3	0.24±0.26	-30.14±2.6	59.26 ± 3.31
WSEP 5	224. 2.7±2.3	0.20±0.06	-29.14±2.8	49.21 ± 3.72
WSEM 1	43.40±2.70	0.30±0.121	-15.3±0.90	46.62 ± 2.82
WSEM 2	62.32±3.70	0.38±0.152	-14±1.533	53.30 ± 1.21
WSEM 3	31.24±0.80	0.33±0.009	-12.7±1.20	44.72 ± 3.13
WSEM 4	30.76±1.29	0.36±0.043	-7.9±1.60	72.82 ± 0.80
WSEM 5	42.70±2.26	0.32±0.047	-11.9±1.60	54.12 ± 1.20

Values are expressed as mean ± standard deviation (n=3). WSEP: *W. somnifera* extract loaded PCL nanoparticles; WSEM: *W. somnifera* extract loaded MPEG-PCL nanoparticles

Nanoparticle structure plays an important role in determining their adhesion and interaction with cells. As it can be observed in the TEM images of the PCL and MPEG-PCL nanoparticles, they have spherical shape (**Figure 6**).

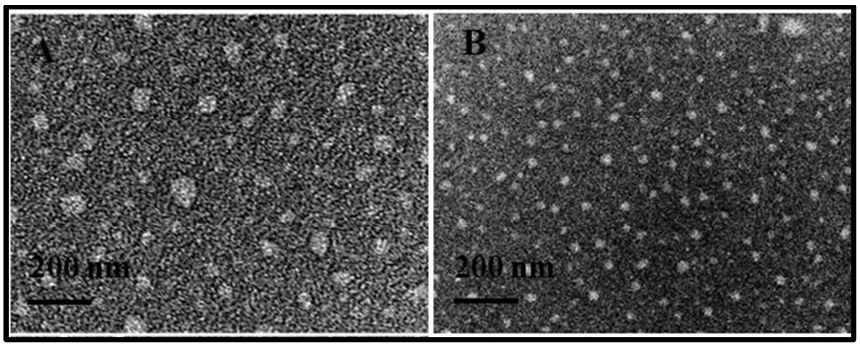

Figure 6. Characterization of *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles. TEM image of *W. somnifera* extract loaded PCL nanoparticles (A), TEM image of *W. somnifera* extract loaded MPEG-PCL nanoparticles (B).

The surface of PCL nanoparticles appears to be rough and porous. The pores surface of PCL/Pluronic F68 and MPEG-PCL/Pluronic F68 nanoparticles could be attributed to the hydrophilicity of Pluronic F68. The surface charge is an important parameter in nanoparticles systems because it can greatly influence particles stability (He et al., 2010). It is also an important factor to determine their *in vivo* interaction with the cell membrane, which is usually negatively charged.

3.4.2.3. Characterization of curcumin nanoparticles

Curcumin encapsulated PCL and MPEG-PCL nanoparticles were prepared by solvent evaporation method. Curcumin and MPEG-PCL were dissolved in acetone then this organic phase was added to an aqueous phase containing surfactant.

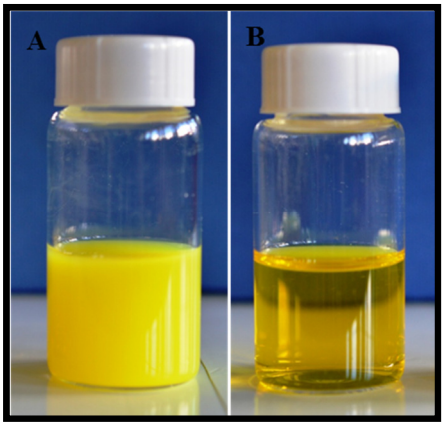

Figure 7. Aqueous solubility of curcumin in PCL nanoparticles (A) MPEG-PCL nanoparticles (B)

In the structure of MPEG-PCL, PEG is the hydrophilic segment and PCL is the hydrophobic segment, so the MPEG-PCL micelles always had a core-shell structure with a PCL core and a PEG shell. Self-assembly of curcumin and MPEG-PCL created a core-shell. It is well know that curcumin is not soluble in aqueous medium, but the prepared nanoparticles had shown better aqueous solubility (**Figure 7**). The size distribution of curcumin loaded PCL and MPEG-PCL nanoparticles were measured by DLS and the size distribution of the nanoparticles are shown in **Figure 8**.

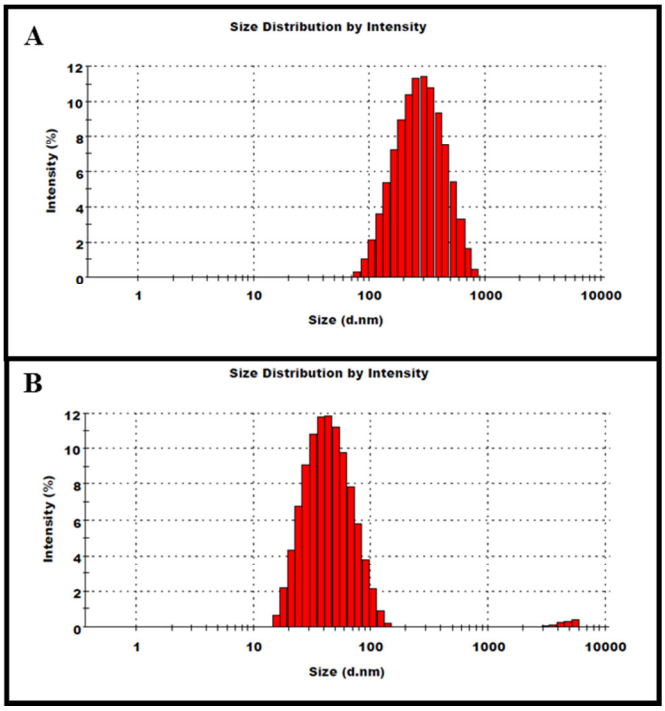

Figure 8. Preparation and characterisation of curcumin loaded PCL and MPEG-PCL nanoparticles. Particles size and distribution of curcumin-PCL nanoparticles (A), Particles size and distribution of curcumin-MPEG-PCL nanoparticles

The polydispersity index and zeta potential of particles are important physico-chemical parameters, which influence the stability of the polymer. The polydispersity index was determined to be approximately 0.22, exhibiting a homogeneous distribution in diameter. Moreover, the lowest zeta potential value (-13.5 mV) of these particles enhances their suitability further, compared to other batches. When the particle size of curcumin encapsulated PCL and MPEG-PCL nanoparticles were compared with the size of the free PCL and MPEG-PCL nanoparticles, a gradual increase in their size could be observed, evidencing of the incorporation of curcumin into the nanoparticles.

The morphology of the nanoparticles was determined by TEM. As seen in the TEM image of curcumin loaded PCL and MPEG-PCL nanoparticles (**Figure 9),** they presented a smooth spherical shape and were within the range of 200-250 nm for PCL and 30-70 nm for MPEG-PCL nanoparticles (Table 4). The results obtained from particles size analyser correlates with the results obtained from TEM.

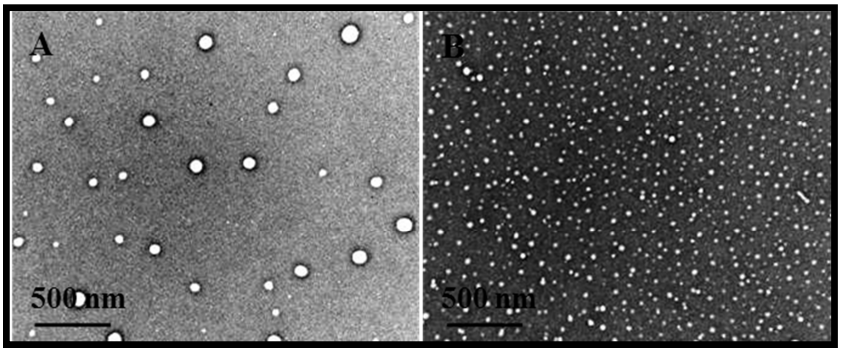

Figure 9. Morphological characterisation of curcumin loaded nanoparticles. TEM image showing spherical shape of curcumin loaded PCL nanoparticles (A) and MPEG-PCL nanoparticles (B)

The average size of the prepared nanoparticles loaded with curcumin ranged between 226-250 nm for PCL nanoparticles, whereas, their MPEG-PCL nanoformulations produced much smaller particles in the size rage of 44-72 nm (**Table 4)**.

Table 4. Particle size, PDI, Zeta potential and Entrapment efficiency of curcumin loaded PCL and MPEG-PCL nanoparticles. Values are expressed as mean ± standard deviation (n=3).

Formulation code	Zeta Average Size (nm ± SD)	Polydispersity index (PI ± SD)	Zeta potential (mV ± SD)	Entrapment efficiency (% ± SD)
CURP1	250.20±2.4	0.32±0.14	-14.6±2.2	39.08 ± 2.15
CURP2	243.16±3.2	0.28±0.20	-26.12±2.1	46.06 ± 3.52
CURP3	232.64±2.6	0.32±0.34	-14.10±3.2	42.16 ± 2.32
CURP4	**224. 28±2.3**	**0.24±0.26**	**-22.24±1.6**	**72.26 ± 3.31**
CURP5	226. 4.7±5.2	0.18±0.16	-17.14±1.8	46.32 ± 2.24
CURM1	66.40±2.72	0.22±0.242	-16.4±0.52	38.12 ± 3.42
CURM2	72.22±3.14	0.26±0.136	-13.2±1.32	42.10 ± 2.32
CURM3	44.24±1.46	0.24±0.018	-11.6±2.4	56.62 ± 4.13
CURM4	**46.18±2.32**	**0.22±0.012**	**-13.5±2.10**	**85.82 ± 1.80**
CURM5	56.26±3.14	0.20±0.062	-13.6±1.12	59.22 ± 2.10

CURP: curcumin loaded PCL nanoparticles; CURM: curcumin loaded MPEG-PCL nanoparticles

3.4.3. *In-vitro* release of *W. somnifera* extract and curcumin nanoparticles

In vitro release behavior of *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles were carried out in PBS (pH 7.4) at 37 °C. **Figure 10** shows the release kinetics of *W. somnifera* extract from PCL and MPEG–PCL nanoparticles for 7 days. The free *W. somnifera* extract released much faster than *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles. In the first 6 hrs both *W. somnifera* extract loaded nanoparticles showed an initial faster release of 30-40% and this might be due to the drug released from the surface of the nanoparticles. In the following period of study 168 hrs, nearly 80% of the total *W. somnifera* extract was released from the PCL and MPEG-PCL nanoparticles. Both WES nanoparticles showed features of controlled release pattern.

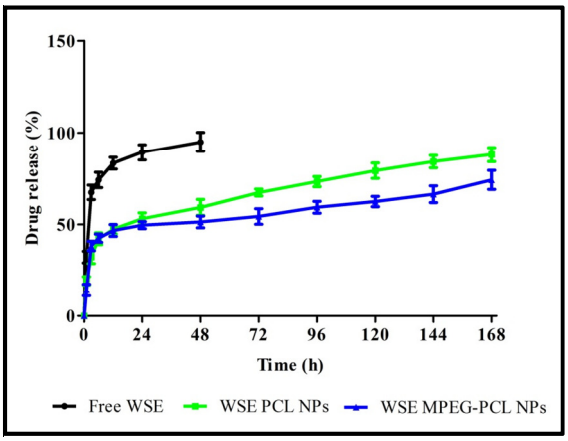

Figure 10. Drug release profile of free *W. somnifera* extract (WSE), WSE loaded PCL (WSE PCL NPs) and WSE loaded MPEG-PCL (WSE MPEG-PCL) nanoparticles

The release profile of free curcumin and curcumin from PCL and MPEG-PCL nanoparticles was studied *in vitro*, and the results are shown in **Figure 11**.

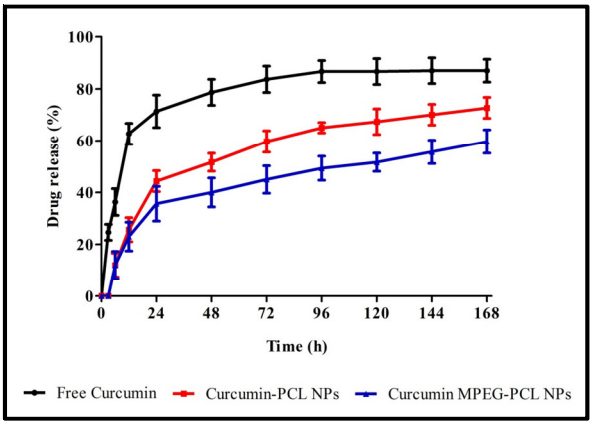

Figure 11. *In-vitro* drug release profile of curcumin from PCL and MPEG-PCL nanoparticles.

Approximately 70% of curcumin was released to the media in free curcumin in 24 hrs, whereas by then, only 26% of the encapsulated curcumin was released from curcumin nanoparticles. Both the PCL and MPEG-PCL nanoparticles exhibited initial burst release followed by a slower sustained release pattern. The initial burst release might be attributed to the diffusion of curcumin from PCL or MPEG-PCL nanoparticles. For instance curcumin MPEG-PCL nanoparticles attained more sustained release compared with PCL nanoparticles. The

controlled release pattern of WES and curcumin PCL and MPEG-PCL nanoparticles might help to achieve better pharmacological dynamics and kinetics.

3.4.4. Differential scanning calorimetry (DSC) analysis for *W. somnifera* extract and Curcumin nanoparticles

DSC analysis was performed to know the physical nature of the drug in the nanoparticles. The DSC is an accurate and rapid thermo analytical technique, widely used by the pharmaceutical industry and in drug research, to investigate several physicochemical phenomena, such as polymorphism, melting and crystallization, purity, drug–excipient interaction, polymer properties. The DSC chromatogram of *W. somnifera* extract shows the melting endothermic peak at 111.26 °C (**Figure 12**). However, no melting peak was detected for both PCL and MPEG-PCL nanoparticle formulations, evidencing the absence of crystalline drug in the nanoparticles, at least at the particle surface level. Therefore, it could be concluded that *W. somnifera* extract in the nanoparticles was in an amorphous or disordered crystalline phase of a molecular dispersion or a solid solution state in the PCL/Pluronic F68 and MPEG-PCL/Pluronic F68 matrix after its production.

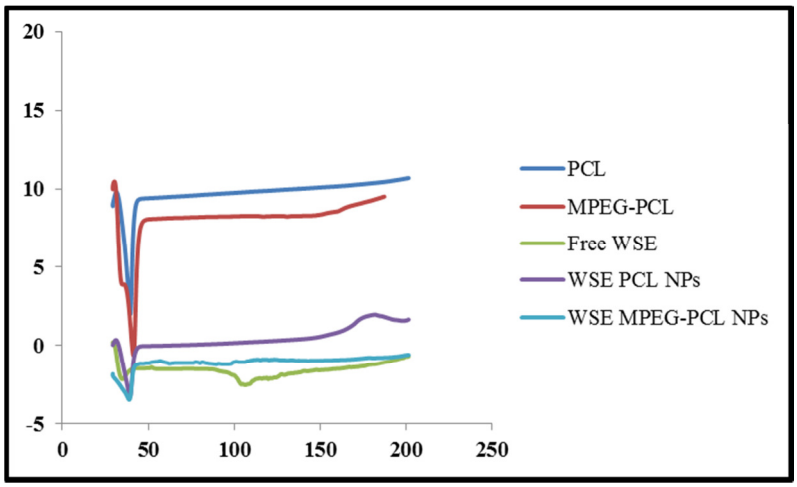

Figure 12. Differential scanning calorimetric thermo gram of *W. somnifera* extract (WSE) loaded PCL and MPEG-PCL nanoparticles.

Free curcumin had an endothermic melting peak at 178.59 ◦ C implying its crystalline state of curcumin (Ghosh et al., 2014). This peak was absent in the thermo gram of curcumin-loaded PCL and MPEG-PCL nanoparticles, which only exhibited the characteristic melting peaks of MPEG-PCL around 55.52 ◦C (**Figure 13**).

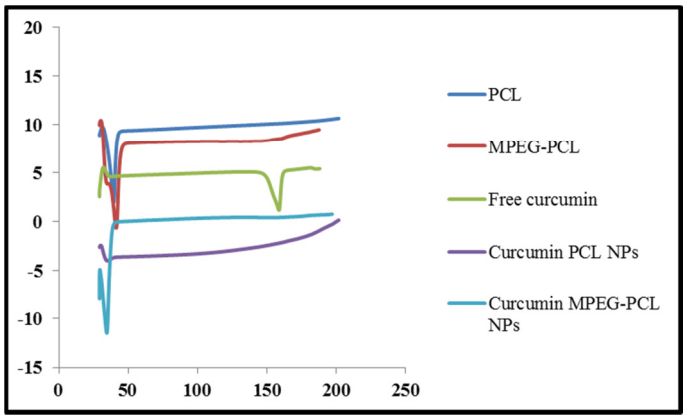

Figure 13. Differential scanning calorimetric thermo gram of PCL and MPEG-PCL loaded curcumin nanoparticles

The absence of detectable crystalline domains of curcumin in the loaded particles indicates that encapsulated curcumin was in the amorphous form in the polymeric matrix. The presence of drug in crystalline form inside nanoparticles can be an obstacle due to the difficulty in the diffusion of large-sized molecules from the small pores of the nanoparticles. However, a sustained release of the encapsulated drug is possible if the drug is in amorphous phase, as it facilitates easy diffusion of drug molecules through the polymeric matrix.

3.4.5. X-ray diffraction (XRD) analysis for *W. somnifera* extract and curcumin nanoparticles

The overlaid XRD pattern of MPEG-PCL, *W. somnifera* extract, *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles is shown in **Figure 14**. The XRD pattern of *W. somnifera* extract exhibited diffraction peaks at 2θ value of 22.40 indicating their crystalline nature. The characteristic crystalline peaks of *W. somnifera* extract was absent in the patterns of WSE loaded PCL and MPEG-PCL nanoparticles suggesting the amorphous state of nanoparticles. Whereas, the XRD pattern of physical mixture diffraction peaks of MPEG-PCL and *W. somnifera* extract reveals an incomplete dissolution and the existence of a crystalline state of *W. somnifera* extract in the polymer.

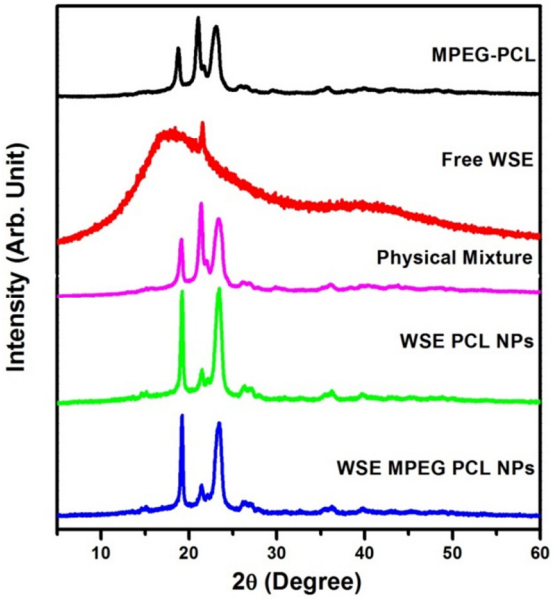

Figure 14. X-ray diffraction patterns of MPEG-PCL, *W. somnifera* extract (WSE), physical mixture and WSE loaded PCL and MPEG-PCL nanoparticle

To examine the crystallinity of nanocurcumin, XRD analysis was carried out. XRD studies on pure curcumin, MPEG-PCL and curcumin-loaded PCL and MPEG-PCL nanoparticles was performed using an X-ray diffractometer (Philips, PW1710) with a horizontal goniometer. The samples were placed in the sample holder and scanned at a rate of 1° min^{-1} from 0° to 60°. MPEG-PCL exhibit diffraction peaks at 2θ value of 19.2°, 21.4° and 23.5° (**Figure 15**).

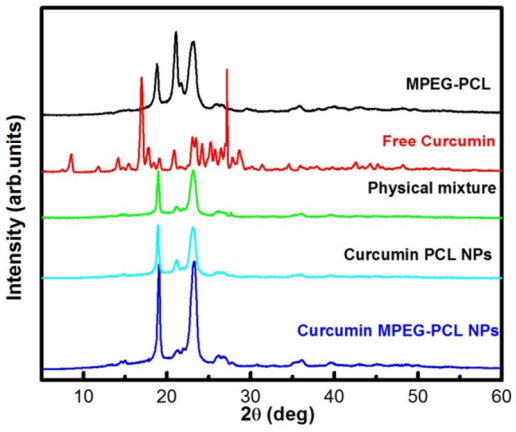

Figure 15. X-ray diffraction patterns of MPEG-PCL, physical mixture, curcumin and curcumin loaded PCL and MPEG-PCL nanoparticles

By comparing the XRD patterns of curcumin and curcumin loaded nanoparticles, it was found that the characteristic crystalline peaks of curcumin was absent in the patterns of curcumin-loaded PCL and MPEG-PCL nanoparticles, which suggests that curcumin was molecularly dispersed and there is no curcumin crystals in the nanoformulations. So it is concluded that the encapsulated curcumin was in amorphous state.

The noticed difference in the particles size between the free and *W. somnifera* extract or curcumin encapsulated nanoparticles is a proof that *W. somnifera* extract or curcumin has been loaded into the polymer matrix. When compare with PCL nanoparticles the MPEG-PCL nanoparticles were much smaller in size and this is probably due to the amphiphilic nature of the polymer. Zeta potential is a physical property present in any particle in dispersion and it is an indirect measurement of the surface charge (Malvern, 2012). Surface charge is important in determining whether the nanoparticles will cluster in blood flow or will adhere to or interact with oppositely charged cell membrane (Danafar et al., 2014; Kumari et al., 2010). Our curcumin encapsulated MPEG-PCL nanoparticles showed the surface charge range between -11 to 16, which indicates higher stability of the nanoparticles. A successful nano carrier system is the one which has the higher entrapment efficacy to reduce the quantity of the carrier required for administration. In our study we got 85.82% of entrapment efficacy for MPEG-PCL nanoparticles.

Conventional oral drug administration does not usually provide rate-controlled release or target specificity. In many cases, conventional drug delivery provides sharp increases of drug concentration at potentially toxic levels. In our experiments free *W. somnifera* extract released in the PBS faster in such a way that within 48 hrs 90% of *W. somnifera* extract release was achieved, when converted as nanoparticles *W. somnifera* extract was released in a controlled manner. This is an added advantage of nanoencapsulation.

3.5. Conclusion

Although, phytomedicines possess excellent *in-vitro* bioactivity, the poor aqueous solubility, larger molecular size and extensive metabolism limits their clinical outcome. Application of nanotechnology for phytomedicine increases their bioavailability and bioactivity by reducing the size of the particles and surface modification.

Nanomaterials aids the targeted delivery, sustained delivery and improves the pharmacokinetics profile, diffusion of drugs into various organs by crossing the barriers including the blood brain barrier. In this study we have prepared *W. somnifera* extract (containing the bioactive compounds of *W. somnifera*) and curcumin encapsulated PCL and MPEG-PCL nanoparticles by solvent displacement method. The size of these nanoparticles was influenced by polymer drug ratio. The prepared nanoparticles were spherical in shape with a smooth surface. The hydrophobicity of *W. somnifera* extract or curcumin was overcome by nanoencapsulation. Furthermore, the prepared nanoparticles can be administered parentally because of their smaller size and biodegradability of the polymer.

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Chapter 4:
In vitro and *in vivo* assays with *W. somnifera* extract and curcumin loaded nanoparticles

4. *In vitro* and *in vivo* assays with *W. somnifera* extract and curcumin loaded nanoparticles

4.1. Abstract

Polymeric nanoparticles prepared and characterized as described in the previous section were evaluated for their cellular uptake, cytotoxicity and neuroprotection in U251 glioma cells *In-vitro* neuroprotective effect was analyzed against *tert*-Butylhydroperoxide (tBHP) induced oxidative damage and *in-vivo* localization was assessed in Zebrafish. *In vitro* cytotoxicity assay with U251 human glioma cells showed that the empty (PCL and MPEG-PCL) nanoparticles were nontoxic to the cells up to 500 µg/ml. Treatment of U251 giloma cells with PCL and MPEG-PCL nanoparticles loaded with curcumin or *W. somnifera* extract revealed efficient cellular uptake. The cellular uptake of MPEG-PCL nanoparticles were higher compare to PCL nanoparticles. The *in-vivo* localization of nanoparticles in Zebrafish model suggested that the MPEG-PCL nanoencapsulation of WSEC (*W. somnifera* extract co-encapsulated with coumarin-6 for localization study) or curcumin had efficient delivery compared with the free drug (*W. somnifera* extract or curcumin) or loaded in PCL nanoparticles. Moreover, MPEG-PCL di-block copolymer loaded *W. somnifera* extract or curcumin nanoparticles was able to protect the cells against tBHP induced oxidative damage in a superior way compared to free *W. somnifera* extract or curcumin. Together, our results show that *W. somnifera* extract or curcumin loaded MPEG-PCL di-block copolymer nanoparticles possess significantly higher neuroprotective effect in U251 human glioma cells compared to the free drugs and *W. somnifera* extract or curcumin encapsulated in PCL nanoparticles.

4.2. Introduction

Medicines derived from plants have played a crucial role in the health care of many cultures of the human history (Gong et al., 2013). Herbal medicine has been used as a remedy for neurological disorders for more than a century and the initial drugs used to treat pathologic conditions of the CNS were based on natural resources specifically from plants (Gomes et al., 2009). After the isolation of active constituents, such as morphine from *Opium* species, the scientific understanding of psychoactive plants has advanced significantly (Sarris et al., 2011). In this context, bio-active compounds from *Hypericum perforatum* (St. John's wort) is another good example for the potential of herbal medicine. Extract/compounds of *H. perforatum* are well recognized in the treatment of mild to moderate depression today in several countries. Recently, it is proven that the bioactive compound from this plant increases the neuroprotective activity and memory in diabetic rats (Hasanein and Shahidi, 2011)

W. somnifera a well-known medicinal plant in Indian ayurvedic medicine, is distributed throughout India. Due to the high medicinal value and nontoxic property of this plant referred as Indian ginseng. The major biochemical constituents of *W. somnifera* are steroidal alkaloids and lactones, together known as withanolides (Ganzera et al., 2003). The isolated bioactive constituent as well as extract of this plant has been reported to possess adaptogenic, anticancer, anti-convulsant, immunomodulatory, anti-oxidative and neurological effects (Chatterjee et al., 2010). Similar to *W. somnifera*, *Curcuma longa* in general, and its natural compound curcumin in specific has been used as a traditional therapeutic agent in Asian medicine to treat various common ailments (Gregory et al., 2014b). Curcumin has been used as food pigment in India and China. It is highly pleiotropic molecule able to interact with large number of molecular target and particularly safer even at higher doses (8g/day) (Gupta et al., 2013). Recent studies have shown that curcumin exhibit multiple pharmacological activities, including anti-inflammatory (Zhang et al., 2015), antioxidant (Motaghinejad et al., 2015), anti-bacteria, anti-virus, and hyperlipidemic activities (Egan et al., 2004).

Although, several studies have explored the pharmacological importance of *W. somnifera* extract and curcumin, their clinical application has been hindered due to several issues. For instance, poor aqueous solubility of *W. somnifera* extract and curcumin leads to the poor cellular uptake, bioavailability that limits the in-vivo pharmacokinetics (Jagetia and Aggarwal, 2007). Hence, we have developed *W. somnifera* extract or curcumin loaded PCL and MPEG-PCL nanoparticles that showed improved water solubility and sustained release profile compared to the free forms. In this study, we have analyzed the cellular uptake and protective effect of those

nanoformulations against tBHP induced oxidative stress in U251 glioma cells. Though there are a number of studies focusing on *in vitro* evaluation of nanoparticles, studies related to *in vivo* toxicity is scarce. In this context, *Danio rerio* (Zebrafish) is currently emerging as an excellent model. Zebrafish is a small benthopelagic tropical freshwater fish, native from India and Southeast Asia. The advantages of using Zebrafish for experimental bioassays include its size, robustness, short life cycle, high fecundity and potential to easily breed in captivity. Hence, in addition to in vitro assays, we have also performed *in vivo* assays with Zebrafish model for evaluate the localization of nanoparticles.

4.2. Methods and materials

4.2.1 Cell culture

Human glioblastoma-astrocytoma epithelial-like cell line (U251) was obtained from the faculty of Pharmacy, University of Porto. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% streptomycin, 10% fetal bovine serum (FBS) and incubated in a carbon dioxide incubator (5% CO_2) at 37 °C.

4.2.2 Cytotoxicity Assay

The effect of *W. somnifera* extract and curcumin in the free form and in the form of nanoparticles on cell proliferation was determined using MTT assay. Briefly, U251 cells were plated onto 24-well flat-bottom plates at a concentration of 10^4 cells per well (Orange Scientific products, Belgium) with standard culture medium. After 24 hrs of incubation at 37 °C, the culture medium was removed and replaced with 500 µl of fresh culture medium augmented with different concentrations of *W. somnifera* extract and curcumin in the free form and as nanoparticles (PCL and MPEG-PCL). To check the influence of polymer, empty PCL and MPEG-PCL nanoparticles were added to the cells in separate experiments. After 48 hrs of treatment, culture medium containing free drugs, drug loaded nanoparticles and empty nanoparticles were removed and replaced with 500 µl of fresh culture medium containing MTT (0.5 mg/ml in Krebs medium, pH.7.4) and further incubated under similar conditions for another 4 hrs. After the 4 hrs incubation, MTT solution was discarded from the wells and 500 µL of DMSO: ethanol (1:1) was added and the plates were shaken for 10 min at room temperature under dark in order to solubilize any formazan crystals formed by the reaction. The absorbance of the formazan solution from varios treatments was measured at 570 nm using a microplate reader (Synergy™ H Multi-mode Microplate Reader,

BioTek Instruments, USA). The amount of MTT that is converted to formazan after incubation with cells corresponds to the number of viable cells.

4.2.3. *In-vitro* cellular uptake of nanoparticles

Human glioma cells (U251) were seeded in 24-well plates with standard culture medium and allowed to attach and grow. After 24 hrs, cells were co-incubated with 0.5 ml of (5 µg/ml) coumarin- 6 loaded PCL and MPEG-PCL nanoparticles. After different periods of incubation (2, 4 and 24 hrs), cells were washed three times in phosphate buffer saline (PBS) to eliminate any PCL or MPEG-PCL nanoparticles that were not up taken by the cells. Then, the cells were fixed in paraformaldehyde and their nuclei were stained in Hoechst dye and photographed under a fluorescent microscope equipped with photography (Olympus 1X71). In the same manner, the cellular uptake of curcumin loaded PCL and MPEG-PCL nanoparticles were analyzed.

4.2.4. Neuroprotection assay

To evaluate the effect of t-BHP in U251 cells we have tested various concentrations of tBHP (100 µM, 0.25, 0.5 and 1mM) and different periods of incubations (1, 2 and 3 hrs). For the neuroprotection study, an optimized concentration (1mM) of tBHP that could induce oxidative damage to the cells was added together with different concentrations of *W. somnifera* extract or curcumin loaded PCL and MPEG-PCL nanoparticles. The percentage of neuroprotection is calculated by the following equitation.

$$\% \text{ of neuroprotection} = \% \text{ of Cell Viability}_{Ext+ins} - \% \text{ of Cell Viability}_{ins}$$

Whereas,

$\text{Cell Viability}_{Ext+ins}$ = % of cell viability with tBHP, and nanoparticles, relatively to the control cells

$\text{Cell Viability}_{ins}$ = % of cell viability with tBHP, relatively to the control

4.2.5. *In-vivo* localization of nanoparticles in Zebrafish

Zebrafish eggs were collected from adult fish bred under standardized conditions and the eggs were observed with a stereo microscope (Nikon Eclipse TS 100) to select healthy embryos. The selected eggs were washed twice with water and incubated at 25° C for 80 hrs in order for them to develop into larval stage and these larvae were used

for the localization experiment. Briefly, 10 healthy embryos were transferred into each well of a 24 well polystyrene multi well plate in quadruplicate. Two different concentrations (0.25 and 1 µg/ml) of both PCL and MPEG-PCL nanoparticles loaded with *W. somnifera* extract and curcumin were tested. After adding the nanoparticles, the plate was incubated for 80 hrs at 28.5 °C. The development of Zebrafish was observed for the next 80 hrs and the images were recorded under a florescent microscope (Olympus IX71, Hamburg, Germany).

4.2.6. Statistical analysis

All experimental parameters were analyzed statistically and graphs were generated using GraphPad 5 software (Prism, USA). Results are expressed as the mean ± standard error of the indicated number of experiments. The significance of the differences between the means was evaluated using the unpaired two-tailed Student's t-test. A difference of $p \leq 0.05$ between values was considered significant.

4.3. Results and discussion

4.3.1. In vitro cytotoxicity of empty nanoparticles

The MTT colorimetric assay is a well-established method for determining the cytotoxic effect of substances (Sylvester, 2011). Since it is important to know whether an eventual the cytotoxic effect is from the polymer or from the drug, first we have studied the cytotoxicity of empty nanoparticles at different concentrations (1, 10, 50, 100, 500 µg). As shown in **Figure 1**, it is clear that both PCL and MPEG-PCL empty nanoparticles were not significantly toxic up to 500 µg/ml. It should be noted that PCL and MPEG-PCL are non-toxic, biodegradable and biocompatible polymers, which are approved by U.S. Food and Drug Administration (FDA).

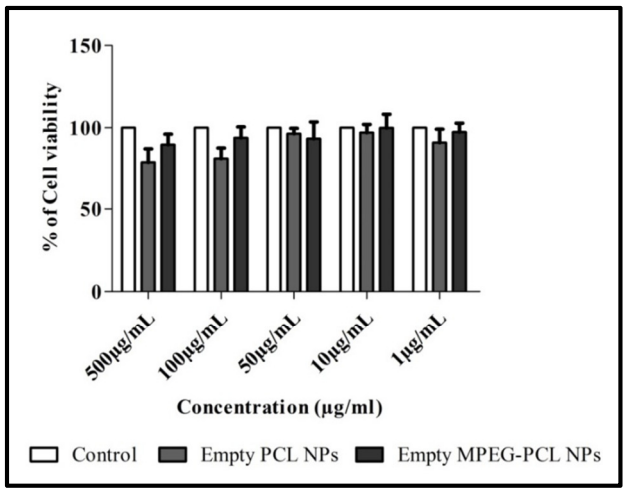


Figure 1. *In- vitro* cytotoxicity of empty nanoparticles in U251 glioma cells (1×10^4 density).

4.3.2. *In- vitro* cytotoxicity of *W. somnifera* extract and curcumin

Among the different concentrations of *W. somnifera* extract tested, the lower concentrations (0.1, 1 and 10 µg/ml) did not affect the cell viability and remained similar to the control cultures. On the other hand, *W. somnifera* extract concentration at 25 µg/ml and above showed significant reduction in cell viability compared to control **(Figure 2)**.

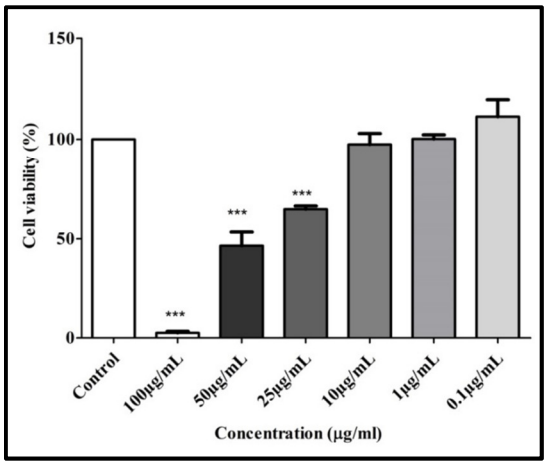


Figure 2. Cytotoxicity of *W. somnifera* extract (WSE) in U251 human glioma cells. Statistical analysis of the data was performed using one sample T-test. Asterisks (*) denotes statistically significant difference from control (only cells) (**P<0.01, ***P<0.001).

Similar to the *W. somnifera* extract, cytotoxic effect of free curcumin was also tested by MTT assay. From previous reports, we found that above 25 µg/ml of curcumin is cytotoxic, so we tested until the maximum concentration of

25 µg/ml. Among the different concentration of curcumin tested, 25 µg/ml showed significant reduction in cell viability compare to the control **(Figure 3).** As observed in the case of *W. somnifera* extract, lower concentrations of curcumin such as 0.1, 1, 5, and 10 µg/ml did not affect cell viability significantly and remained similar to the control.

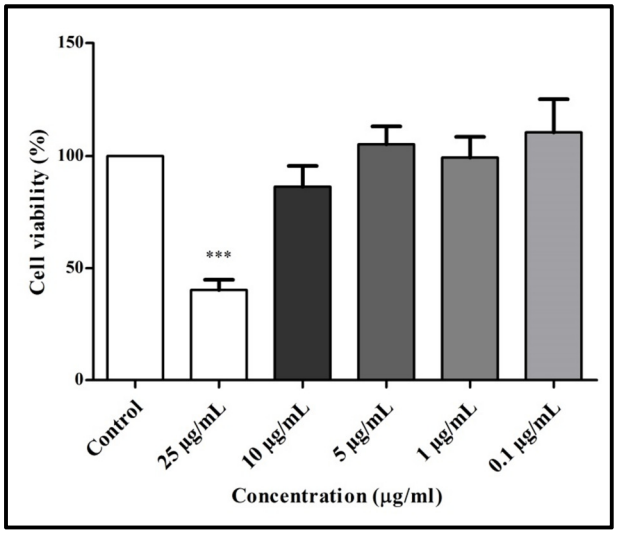


Figure 3. Cytotoxicity of curcumin in U251 human glioma cells (1×10^4 density). Statistical analysis of the data was performed using one sample T-test. Asterisks (*) denotes statistically significant difference from control (only cells) (**P<0.01, ***P<0.001).

From these results it is clear that *W. somnifera* extract and curcumin inhibit cells viability in a concentration dependent manner.

4.3.3. *In-vitro* cytotoxicity *W. somnifera* extract and curcumin loaded nanoparticles

As observed for free *W. somnifera* extract, 25 µg/ml of *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles significantly inhibited the viability, whereas the lower concentrations (0.1, 1 and 10 µg/ml) did not show any sign of toxicity of cells compared to control. Similar to free WSE, nanoparticles loaded with *W. somnifera* extract also inhibited cell viability in a dose dependent manner (**Figure 4A)**. On the other hand, 10 µg/ml and 25 µg/ml concentrations of curcumin loaded PCL and MPEG-PCL nanoparticles showed significant reduction in cell viability compared to control **(Figure 4B)**. Approximately 50% inhibition of cell viability was observed at 25 µg/ml concentration of curcumin delivered in the form of PCL and MPEG-PCL encapsulated nanoparticles. When the other concentrations of curcumin loaded PCL and MPEG-PCL nanoparticles (0.01, 0.1, and 1 µg/ml) were employed, the numbers of viable cells were almost similar to the control and the small variations observed with

these concentrations are not statically significant. Hence 0.1, 1 and 10 µg/ml of *W. somnifera* extract and curcumin encapsulated PCL and MPEG-PCL nanoparticles were chosen for further assays regarding neuroprotection.

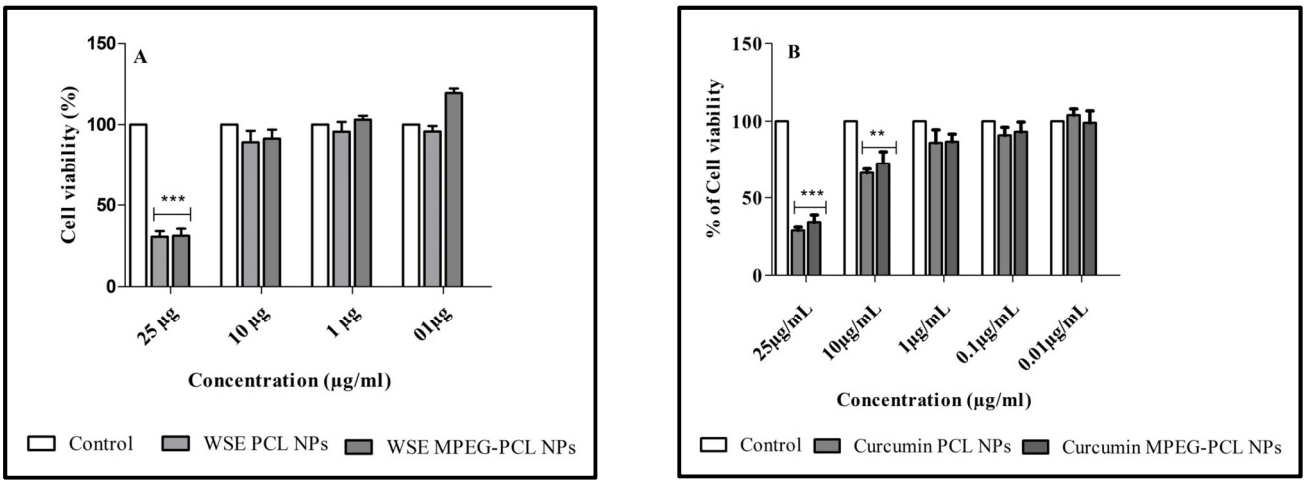


Figure 4. (A) Cytotoxicity of *W. somnifera* extract (WSE) loaded PCL and MPEG-PCL nanoparticles in U251 human glioma cells (1×10^4 density). Data presented (mean ± SD, n = 3). (B) Cytotoxicity of curcumin nanoparticles in U251 human glioma cells. Data presented (mean ± SD, n = 3). Statistical analysis of the data was performed using one sample T-test. Asterisks (*) denotes statistically significant difference from control (only cells) (***P<0.001).

4.3.4. *In-vitro* cellular uptake of *W. somnifera* extract or curcumin nanoparticles

4.3.4.1 In-vitro cellular uptake of W. somnifera extract loaded nanoparticles

The intracellular uptake of *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles were studied by using coumarin-6 co-encapsulation with *W. somnifera* extract (WSEC) (Gao et al., 2013). As seen in **Figure 5,** cells treated with WSEC loaded MPEG-PCL nanoparticle showed profound fluorescence intensity compared to the cells incubated with the similar PCL nanoparticles at equivalent concentration (10 µg/ml), indicating that the WSEC loaded MPEG-PCL nanoparticles should have been up taken more effectively than that of PCL nanoparticles. Compared to the free WSEC, its PCL and MPEG-PCL nanoformulations were up taken by the glioma cells as less as in 2 hrs. Plausibly, the cells must have up taken the particles through endocytosis, which had been confirmed in many other *in vitro* and *in vivo* studies (Carroll et al., 2010; Ding et al., 2011).

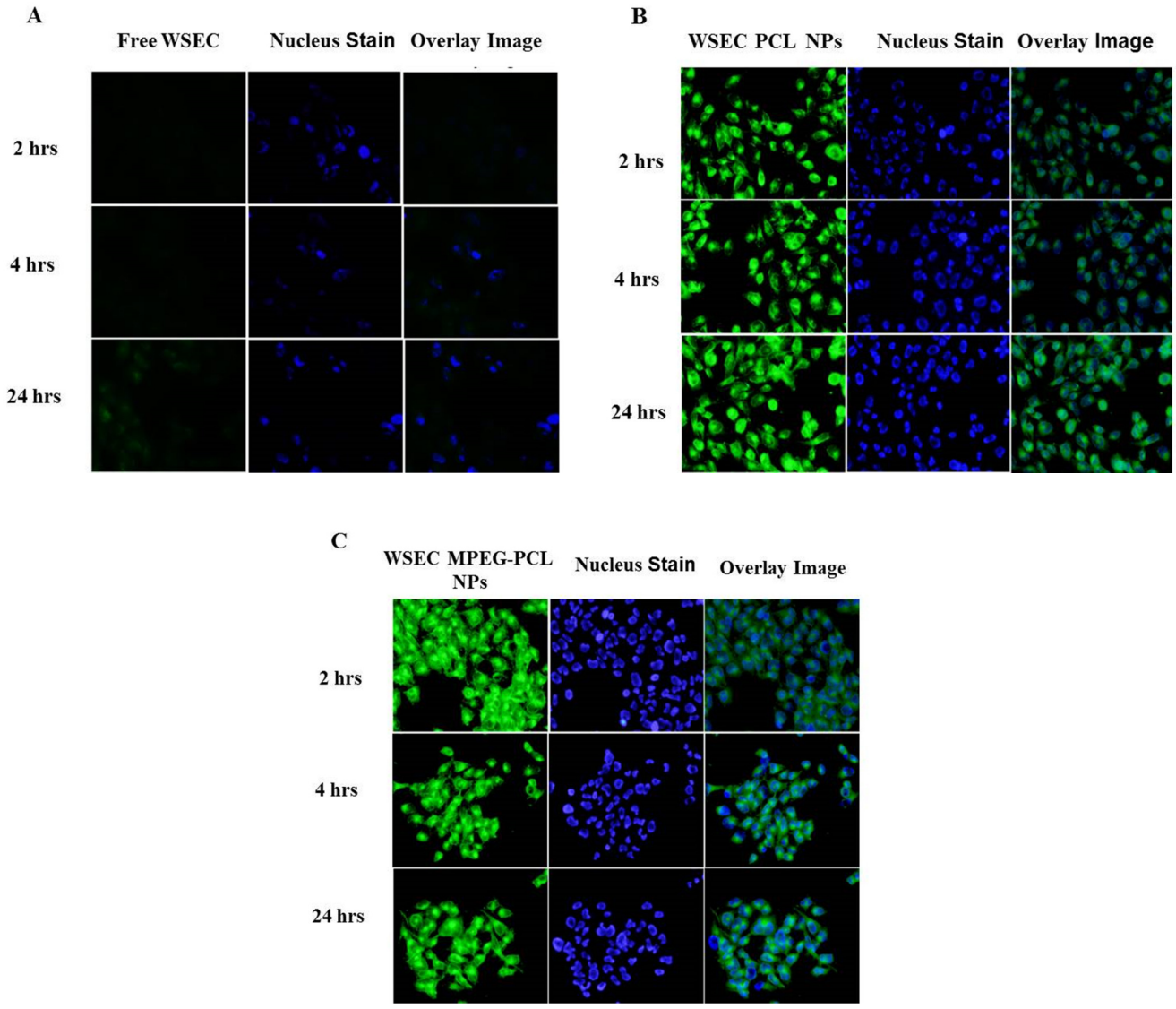
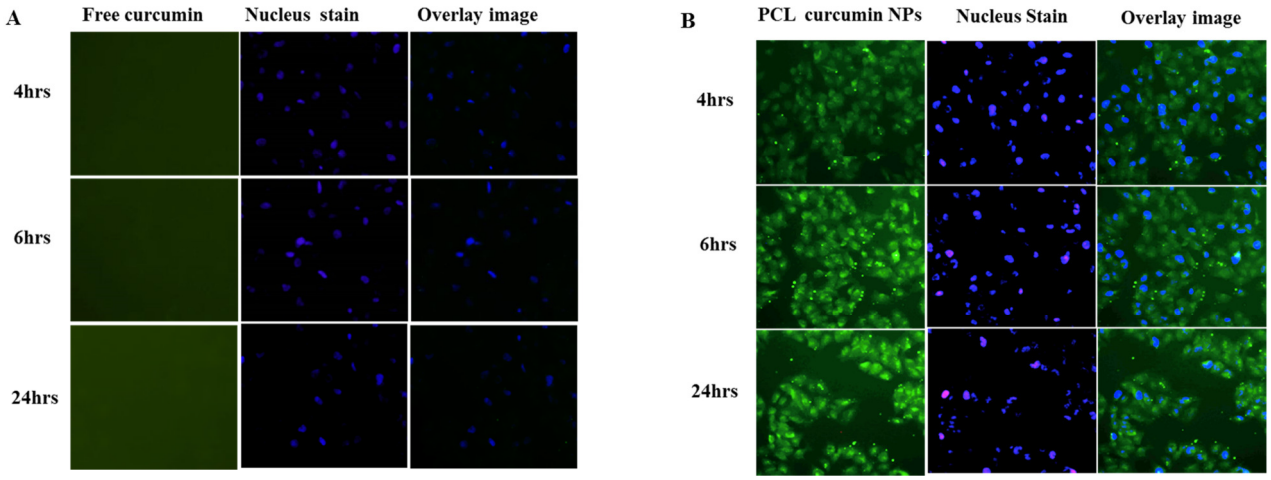


Figure 5. Human glioblastoma cells (U251) incubated with WSEC loaded PCL and MPEG-PCL nanoparticles for different time durations (2, 4, and 24 hrs). After treatments, the cells were analyzed for their fluorescence intensity under fluorescence microscope. Fluorescent images of free WSEC (A), WSEC loaded PCL nanoparticles (B) and WSEC loaded MPEG-PCL nanoparticles (C) showing the internalization of nanoparticles by the U251 human glioma cells.

4.3.4.2. In-vitro cellular uptake of curcumin encapsulated nanoparticles

Curcumin is an auto fluorescent compound that emits a green fluorescence when viewed under a 535–600 nm filter (Ucisik et al., 2013). Following 4, 6 and 24 hrs of treatments of U251 cells with free curcumin and curcumin loaded PCL or MPEG-PCL nanoparticles, the green fluorescence intensity increased in a time-dependent manner.

While cells incubated with free curcumin failed to register any fluorescence even after 24 hrs, the cells treated with curcumin nanoparticles showed fluorescence, indicating that nanoformulation always improves the cellular uptake of curcumin. Maximum fluorescence intensity for each treatment was observed in cells that were undergone 24 hrs co-incubation. MPEG-PCL nanoparticle treated cells showed the highest intensity of fluorescence compared to PCL nanoparticles (**Figure 6 A, B and C**), clearly revealing the importance of MPEG conjugation for highest cellular uptake of nanoparticles. Interestingly, the fluorescence signal displayed by curcumin loaded PCL nanoparticles was more superficial and lighter than the one generated by curcumin-MPEG-PCL, suggesting that the superficial signal might be from the nanoparticles attached to the surface of the cells and the lighter intensity could be because of curcumin dispersed in the cytoplasm rather than accumulated in the nuclei. On the other hand, cells treated with curcumin-MPEG-PCL NPs showed fluorescence not only in the cytoplasm but also in the nuclei, overall increasing the intensity of fluorescence. Since, MPEG-PCL nanoparticles had shown higher amount of fluorescent intensity, we conclude that the attachment of MPEG to PCL has increased the cellular uptake of particles. Overall, curcumin in the form of MPEG-PCL nanoparticles is an efficient way to penetrate cells and get into the cytoplasm and nucleus.



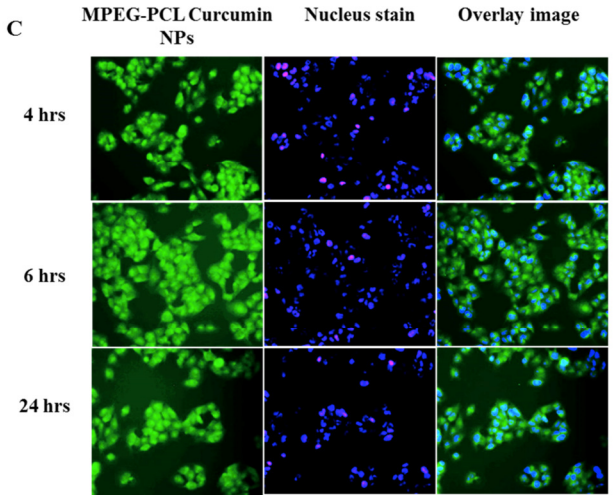


Figure 6. Human glioblastoma cells (U251) incubated with curcumin solution, and curcumin loaded PCL and MPEG-PCL nanoparticles for different time periods (4, 6 and 24 hrs) were analyzed for their fluorescence intensity under fluorescence microscope. Fluorescent images of free curcumin (A), curcumin loaded PCL nanoparticles (B) and curcumin loaded MPEG-PCL nanoparticles (C) showing the internalization of nanoparticles by the U251 human glioma cells.

4.3.5. Neuroprotective effect of *W. somnifera* extract and curcumin PCL and MPEG-PCL nanoparticles

Neurodegenerative diseases are multifactorial pathogenesis characterized by the deregulation of several pathways (Sheikh et al., 2013). Genetic, environmental and endogenous factors related to aging contribute to neurodegeneration, but their pathogenic role and their basic molecular mechanisms are not fully understood (Jellinger, 2010). Free radical mediated injury is known to play a major role in the disease process of ischemic, traumatic, and degenerative disorders in the CNS (Uttara et al., 2009). Therapeutic agents that can target multiple pathophysiological mechanisms can be extremely useful to protect the neuronal cells from damage. Thus a special emphasis was given to test the effect of our nanoformulations on oxidative stress was undertaken.

4.3.5.1. Effect of tBHP concentration in U251 glioma cells

Tert-butyl hydroperoxide (tBHP) is a ROS generating agent that causes lipid peroxidation, which is used as a model substance for the induction of oxidative damage to the cells (Dumont et al., 2002). tBHP has been known to decrease cellular proliferative life span and increase the proportion of cells' positive senescence-associated enzyme activity (Shin et al., 2009). Thus, tBHP is known for the induction of oxidative stress-induced premature senescence.

Neurons are particularly rich in polyunsaturated fatty acids, which make neurons more susceptible to oxidative stress than other cells. tert-Butyl hydroperoxide (tBHP) is commonly used to induce oxidative stress in numerous cell lines including neurons (Hughes et al., 2014). In order to optimize tBHP concentration that could induce oxidative damage in the cells, we have analysed a range of concentrations of tBHP (100 µM, 0.25 mM, 0.5 mM and 1 mM) with different time period of incubation (**Figure 7**). Our results revealed that 1mM tBHP induced optimum oxidative damage to the U251 glioma cells upon 3 hrs incubation. Hence, 1mM tBHP concentration and incubation time of 3 hrs were selected for further neuroprotection experiments.

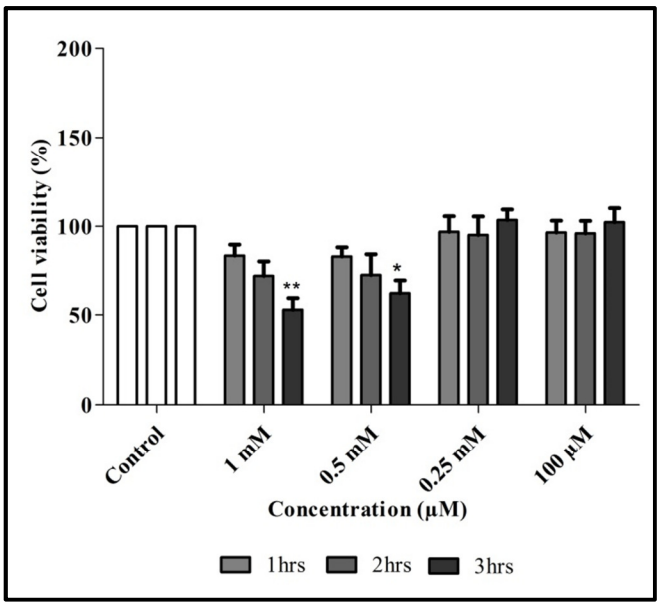


Figure 7. Cell viability analysis by MTT reduction test. U251 cells were incubated with different concentrations of tBHP (100 µM, 0.25, 0.5 and 1mM) for different time period (1, 2 and 3 hrs). Note: 1mM tBHP treatment results in 50% viability reduction in U251 cells upon 3 hrs incubation.

4.3.5.2. Neuroprotective effect of W. somnifera extract loaded PCL and MPEG-PCL nanoparticles

Three different concentrations (0.1, 1 and 10 µg/ml) of *W. somnifera* extract, equivalent *W. somnifera* extract loaded PCL and MPEG-PCL nanoparticles and both PCL and MPEG-PCL empty nanoparticles were analyzed for their neuroprotective potential. Empty nanoparticles did not show any protective effect against oxidative damage, when compared with control cells (**Figure 8**). However, *W. somnifera* extract and *W. somnifera* extract loaded nanoparticles significantly protected neuronal cells against oxidative damage induced by tBHP (**Figure 8 A, B and C)**. When compared with the free *W. somnifera* extract, nanoparticles loaded with *W. somnifera* extract showed better protective effect. Among the nanoparticle formulations, *W. somnifera* extract loaded MPEG-PCL

nanoparticles showed higher percentage of neuroprotection (42.2%) for 0.1 µg/ml, (88.8%) for 1 µg/ml and (95.2%) for 10 µg/ml. Comparatively, *W. somnifera* extract loaded PCL nanoparticles showed less neuroprotective effect of (33.2%) for 0.1 µg/ml, (48.2%) for 1 µg/ml and (56.5 %) for 10 µg/ml respectively.

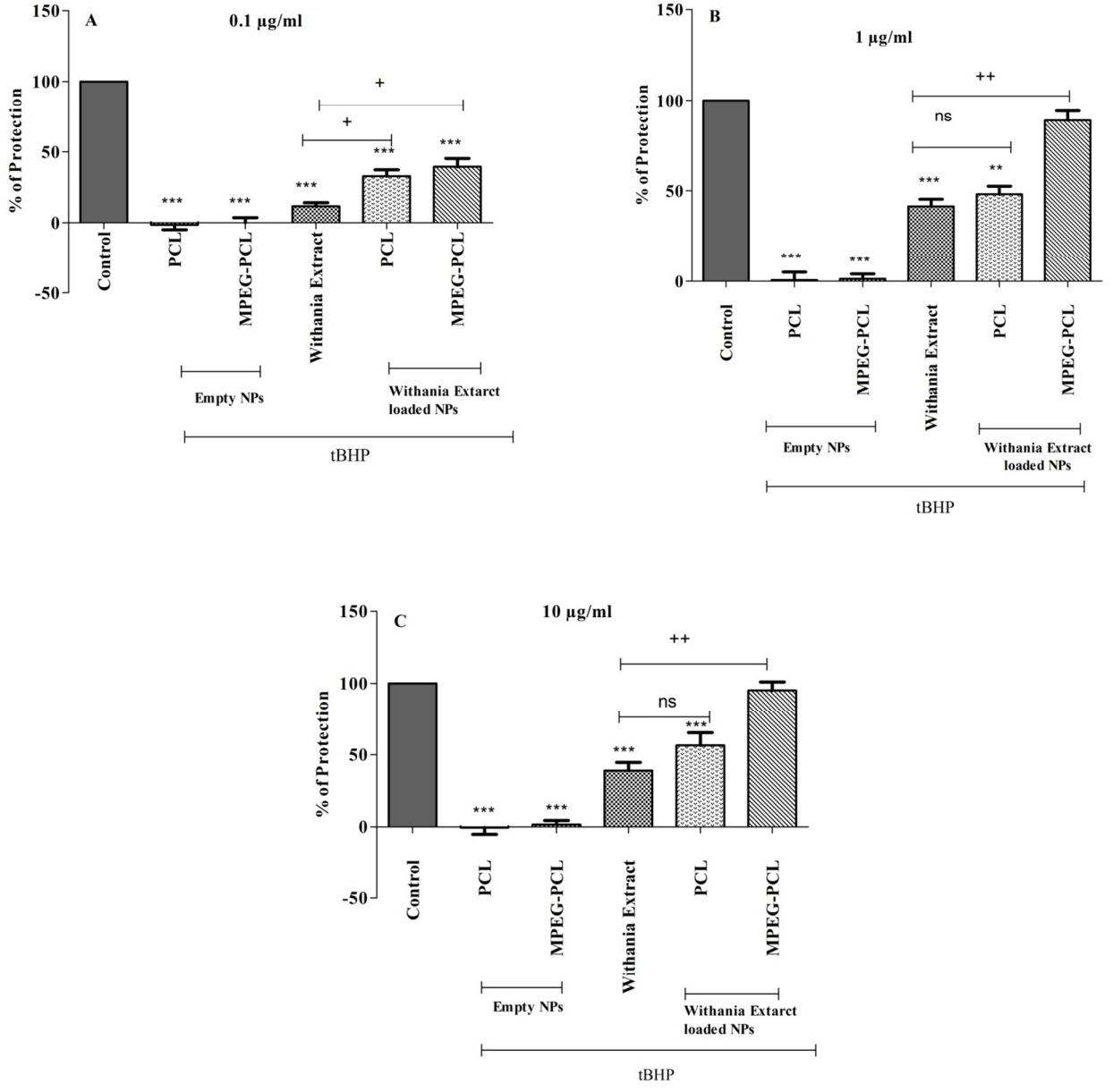


Figure 8. Neuroprotective effect of *W. somnifera* extract (WSE) at different concentrations (0.1 µg/ml, 1 µg/ml and 10 µg/ml) loaded MPEG-PCL and PCL nanoparticles upon 1mM tBHP induced oxidative stress in U251 glioma cells. Data presented (mean ± SD, n = 3). Statistical analysis of the data was performed using non parametric T-test. Asterisks (*) denotes statistically significant difference from free WSE (**P<0.01, ***P<0.001).

As revealed in this experiment (Figure 8), free *W. somnifera* extract could provide neuroprotection to U251 cells against tBHP induced oxidative stress. However, this protection was less effective compared to the equivalent amount of its nanoformulation at all concentrations tested. Particularly, these nanoparticles could protect cells from oxidative damage even at lower doses and this property could be attributed to their water solubility and increased cellular uptake. The protective effect of *W. somnifera* extract nanoparticles seen in the above conditions may be due to the enhanced transport of nanoparticles into glial cells and the sustained release of withanolides, contributing to a suitable concentration of withanolides to neutralize the free radicals formed in neuronal cells. Previous reports also supports the current findings not only in terms of cellular uptake efficiency, but also protection of cells by drug-loaded nanoparticles in several models. Doggui and co-workers reported that packing of curcumin into a PLGA nanoparticles increased its protective effect in neurons against oxidative damage caused upon Alzheimer's disease (Doggui et al., 2012). Tiwari and their group reported on the improved neuroprotective efficacy of nicotine encapsulated PLGA nanoparticles by enhancing the bioavailability of nicotine and subsequent modulation in the indicators of oxidative stress and apoptosis (Tiwari et al., 2013). Similarly, loading of sesamol in solid lipid nanoparticles is reported as an effective strategy to mitigate intra cerebroventricular streptozotocin induced neuronal dysfunction and memory deficits (Sachdeva et al., 2015).

4.3.5.3. Neuroprotective effect of curcumin loaded PCL and MPEG-PCL nanoparticles

Neuroprotective effect of curcumin and curcumin loaded PCL and MPEG-PCL nanoparticles was analyzed against 1mM tBHP induced oxidative stress in U251 cells. Three different concentrations (0.1 µg/ml, 1 µg/ml and, 10 µg/ml) of curcumin, equivalent curcumin encapsulated PCL and MPEG-PCL nanoparticles were co-incubated with the oxidant agent, tBHP for 3 hours. Empty PCL and MPEG-PCL nanoparticles were also tested in the same manner.

Both PCL and MPEG-PCL empty nanoparticles did not show any protective effect against the induced oxidative stress. As expected, bothe curcumin and curcumin loaded nanoparticles showed significant neuroprotection against oxidative damage induced by tBHP (**Figure 9**). When compared with free curcumin nanoparticles showed better protective effect. However, there was a difference between the nanoformulations in terms of protection. Curcumin delivered in the form of MPEG-PCL encapsulated nanoparticles showed higher percentage of protection (79.81%, 84.57% and 37.27% respectively at 0.1 µg/ml, 1 µg/ml and 10 µg/ml concentrations) than equivalent PCL nanoparticles (52.87%, 38.63% and 24.03% respectively at 0.1 µg/ml, 1 µg/ml and 10 µg/ml).

Interestingly, the neuroprotective effect of both the free and nanoformulations of curcumin was reducing with increasing concentrations, suggesting that this compound is more effective as antioxidant at very low concentrations.

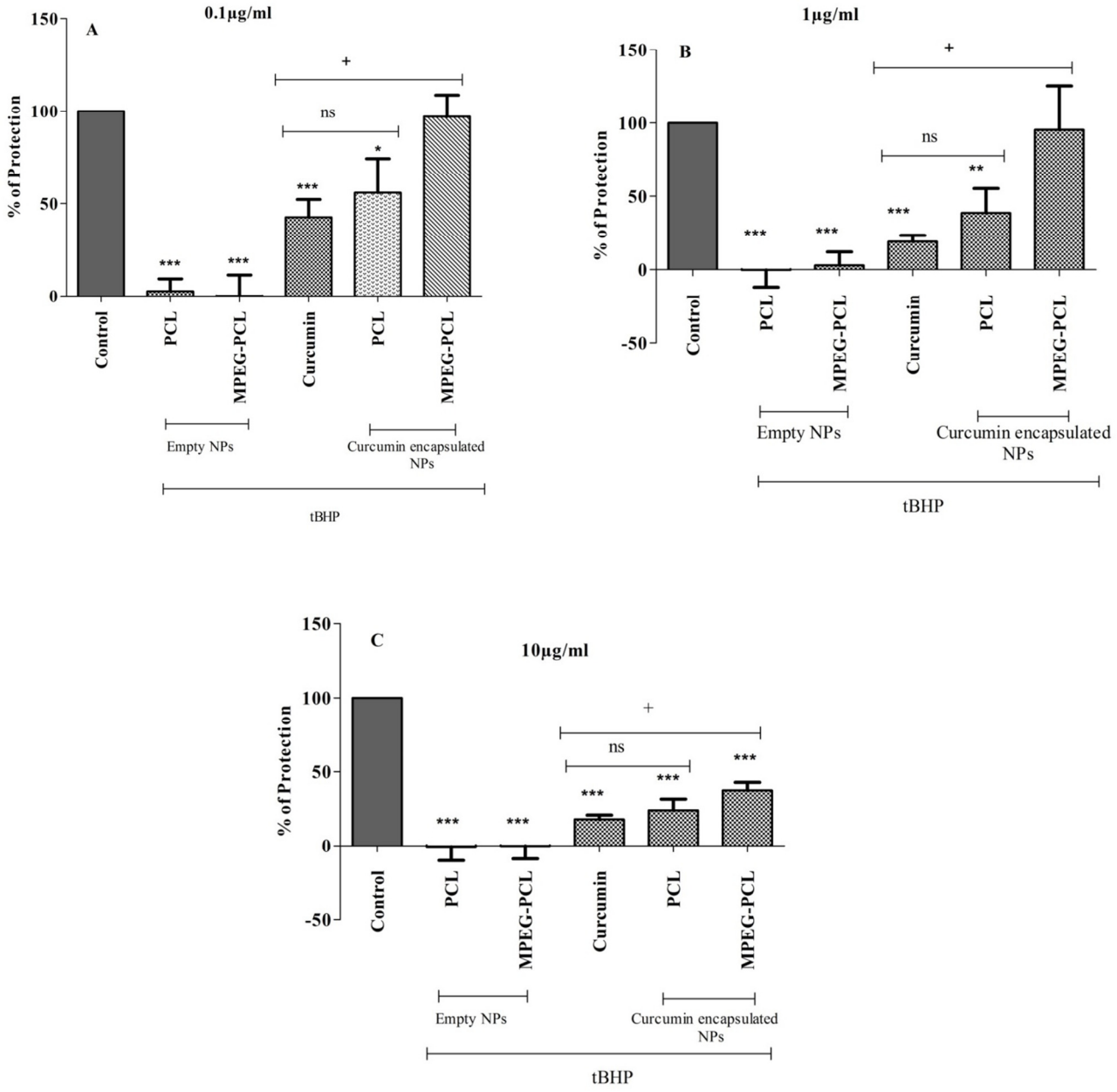


Figure 9. Neuroprotective effect of different concentrations of curcumin loaded MPEG-PCL and PCL nanoparticles (0.1 µg/ml, 1 µg/ml and 10 µg/ml) against tBHP induced oxidative stress in U251 glioma cells. Data presented (mean ± SD, n = 3). Statistical analysis of the data was performed using non parametric T-test. Asterisks (*) denotes statistically significant difference from free curcumin (*P<0.05, **P<0.01, ***P<0.001).

Although many studies have been reported on the effectiveness of curcumin nanoformulations against cancer cell lines, studies pertaining to neuroprotection are very limited. Our study reveals that neuroprotective and antioxidant activities of curcumin could be improved by nanoencapsulation. Curcumin encapsulated in nanoparticles provide neuroprotection from tBHP induced oxidative stress at very low concentration probably due to the enhanced transport of curcumin into cells and the sustainable release of antioxidants from nanoparticles, which should have contributed to a suitable concentration of curcumin to neutralize the free radicals formed in glioma cells. Improved activity of curcumin as nanoformulations has been previously reported. For instance, the cellular uptake of curcumin improved when it was prepared as cationic chitosan/PCL nanoparticles (Liu et al., 2012). Recently, curcumin was successfully encapsulated into polymeric nanoparticles and nanocurcumin showed grater result than free curcumin against different cancer cell lines (Mohanty and Sahoo, 2010).

4.4. *In-vivo* localization of *W. somnifera* extract and curcumin nanoparticles in Zebrafish

Zebrafish has been recognized widely as a model species for developmental biology, gerontology assessment, behavioral ecology, genetics research, and ecotoxicology (Berry et al., 2007). Zebrafish embryos are transparent which permits the imaging of internal organs, easy to assess any alterations. Because of its similarity with human development and at the level of genes, Zebrafish is highly appreciated as a model system to study early development and human diseases. More recently, it has caught the attention of researcher as a perfect model for large scale toxicology screenings. Taking advantage of optically clear Zebrafish embryos, in this study we have utilized this animal model to visualize the internalization nanoparticles *in vivo*.

4.4.1. *In-vivo* localization of nanoparticles in Zebrafish

Animals treated with water (Control) and empty nanoparticles did not show any fluorescence throughout the study period **(Figure 10 A-F)**. On the other hand, the animals treated with free WSEC and WSEC loaded nanoparticles showed faint green fluorescence (**Figure 10 G-L)**. Fluorescence intensity of animals treated with WSEC loaded MPEG-PCL nanoparticles was significantly higher compared to coumarin-6 loaded PCL nanoparticles and free coumarin (**Figure 10 K-L**). Importantly, WSEC nanoparticles were distributed uniformly throughout the animal. Collectively, our results indicate that MPEG-PCL nanoencapsulation is highly efficient in terms of Zebrafish uptake.

The amphiphilic nature, smooth surface and uniform size of MPEG-PCL nanoparticles might have facilitated its effective uptake by the animals (Shen et al., 2015). As it was reported before, PEG has been used as agent for

bacterial/yeast transformation (Karas et al., 2013). In this context, PEGylation could have enabled brain permeability, allowing the accumulation of MPEG-PCL nanoparticles inside the central nervous system more than PCL nanoparticles. It is rational to think that more the uptake of drug loaded nanoparticles, better the neuroprotective activity.

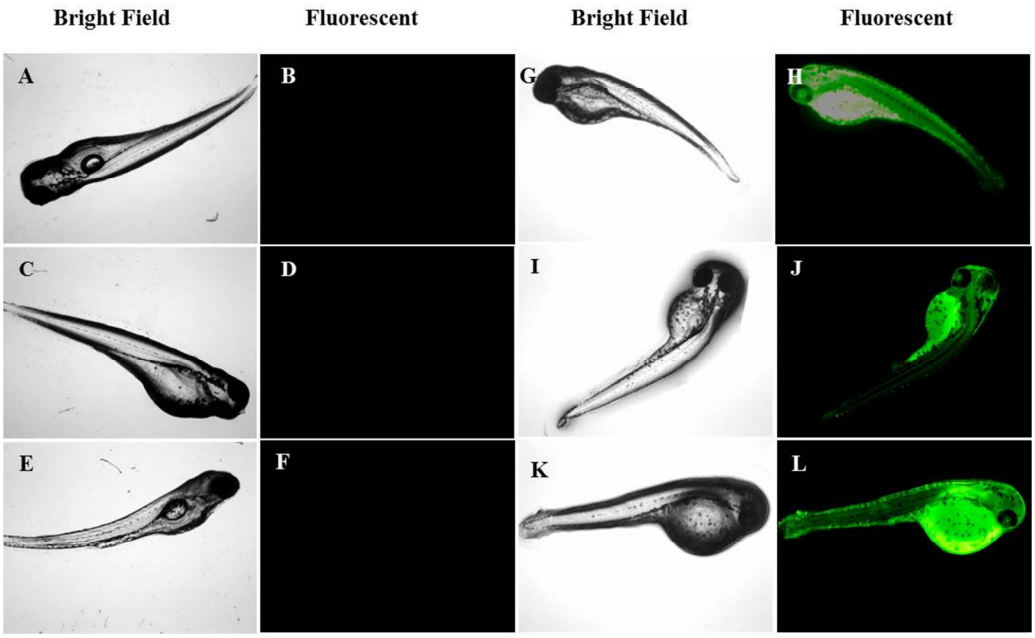


Figure 10. Accumulation of *W. somnifera* extract and coumarin-6 co-encapsulated (WSEC) nanoparticles in 80 hrs old Zebrafish larvae. The images were taken after 4 hrs of incubation with free WSEC and WSEC loaded nanoparticles. Light-fluorescent microscopic views of control Zebrafish (A-B). Light-fluorescent microscopic views of Zebrafish incubated with empty PCL nanoparticles (C-D). Light-fluorescent microscopic views of Zebrafish incubated with empty MPEG-PCL nanoparticles (E-F). Light-fluorescent microscopic views of Zebrafish incubated with free WSEC (G-H). Light-fluorescent microscopic views of Zebrafish incubated WSEC loaded PCL nanoparticles (I-J). Light-fluorescent microscopic views of Zebrafish incubated with WSEC loaded MPEG-PCL nanoparticles (K-L).

4.4.2. *In-vivo* localization of curcumin nanoparticles

Localization of curcumin in the free and nanoparticle forms (PCL and MPEG-PCL) were analyzed in Zebrafish larvae as described above. No fluorescence was detected in the animals treated with water (Control) and empty nanoparticles **(Figure 11 A-F)**. The animals treated with free curcumin and curcumin loaded PCL nanoparticles

showed very low level of fluorescence (**Figure 11 G-J**). However, the fluorescence intensity was significantly higher in the animals treated with curcumin loaded MPEG-PCL nanoparticles compared to free curcumin and its PCL formulation (**Figure 11 K-L**). Moreover, as seen in the **Figure 11L**, the MPEG-PCL nanoparticles were distributed uniformly throughout the animal.

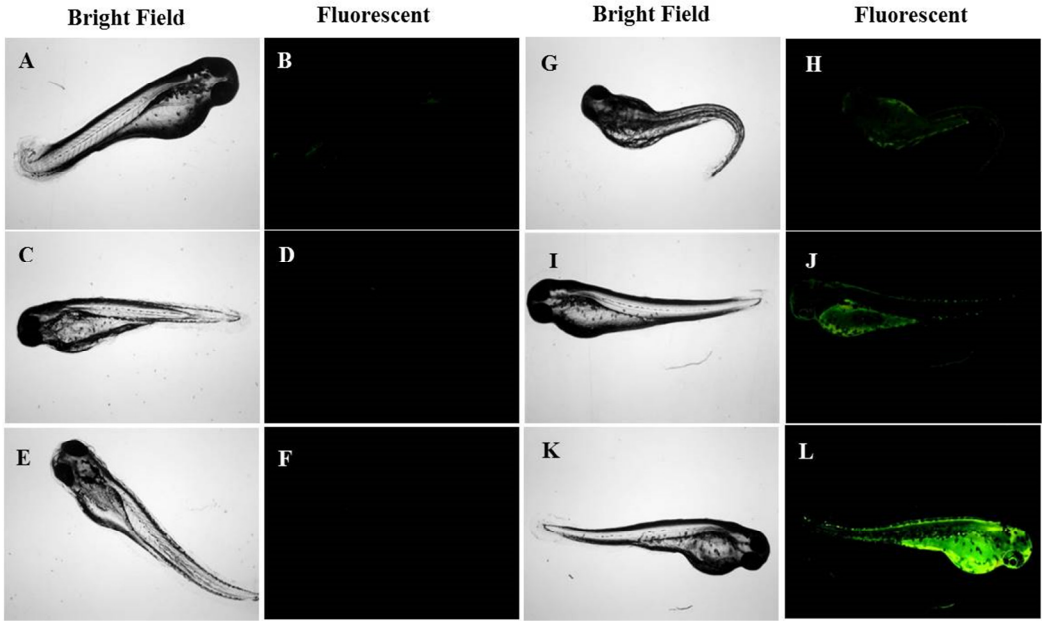


Figure 11. Accumulation of curcumin nanoparticles in 80 hrs developed Zebrafish larvae. The images were taken after 4 hrs incubation with free curcumin or curcumin loaded nanoparticles. Light and fluorescent microscopic views of control Zebrafish (A-B). Light and fluorescent microscopic views of Zebrafish incubated with empty PCL nanoparticles (C-D). Light and fluorescent microscopic views of Zebrafish incubated with empty MPEG-PCL nanoparticles (E-F). Light and fluorescent microscopic views of Zebrafish incubated with free curcumin (G-H). Light and fluorescent microscopic views of Zebrafish incubated curcumin loaded PCL nanoparticles (I-J). Light and fluorescent microscopic views of Zebrafish incubated with curcumin loaded MPEG-PCL nanoparticles (K-L).

Accumulation of nanoparticles in the CNS is of particular interest for brain delivery of drug. Careful analysis revealed that the MPEG-PCL nanoparticles accumulated in the CNS in more than PCL nanoparticles. Collectively, our results indicate that MPEG-PCL nanoencapsulation is highly efficient in term of CNS uptake, which is in accordance with the previous report on the enhanced brain delivery of tanshinone IIA through PEGylation (Liu et al., 2013).

4.5. Conclusion

We found that the antioxidant and neuroprotective effects of *W. somnifera* extract and curcumin could be significantly improved via nanoencapsulation. Among the two different polymers used for the nanoparticle preparation, MPEG-PCL promoted cellular uptake and thereby neuroprotection. In-vivo localization analysis of nanoparticles in Zebrafish suggests that the accumulation of MPEG-PCL nanoparticles is much higher in CNS compared to PCL nanoparticles. Together, our results conclude that MPEG-PCL nanoencapsulation enhances the cellular uptake and neuroprotection of both *W. somnifera* extract and curcumin. This could be a potential candidate for protecting the neurons from oxidative damage.

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Chapter 5:
Antimicrobial activity of cream incorporated with silver nanoparticles biosynthesized from *Withania somnifera*

5. Antimicrobial activity of cream incorporated with silver nanoparticles biosynthesized from *Withania somnifera*

5.1 Abstract

In the present study, we report on the antimicrobial activity of a cream formulation of silver nanoparticles (AgNPs) biosynthesized using *W. somnifera* extract. Aqueous extracts of leaves promoted efficient green synthesis of AgNPs compared to fruits and root extracts of *W. somnifera*. Biosynthesized AgNPs were characterized for their size and shape by physical-chemical techniques such as UV-Visible spectroscopy, Laser Doppler anemometry, Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), X-ray diffraction (XRD) and X-ray energy dispersive spectroscopy (EDX). After confirming the antimicrobial potential of AgNPs, they were incorporated into a cream. Cream formulations of AgNPs and $AgNO_3$ were prepared and compared for their antimicrobial activity against human pathogens (*Staphylococcus aureus, Pseudomonas aeruginosa, Proteus vulgarius, Escherichia coli and Candida albicans*) and a plant pathogen (*Agrobacterium tumefaciens*). Our results show that AgNPs cream possess significantly higher antimicrobial activity against the tested organisms

5.2. Introduction

Nanotechnology and nano-tools have gained much attention due to their wide range of applications in physics, chemistry, biology, material science and medicine (Karuppiah and Rajmohan, 2013). Metal nanoparticles like silver, gold, copper have been used for diagnostic and treatment of disease because of their catalytic, optical, electronic, antimicrobial and magnetic properties(Zayed et al., 2012). Sliver nanoparticles (AgNPs) are generally synthesized by physical and chemical methods such as electrochemical reduction, and thermal evaporation(Jagtap and Bapat, 2013; Tan et al., 2002; Devaux et al., 1993) . But these methods are time-consuming and difficult to scale up. Furthermore these methods are environmentally unsafe due to the use of toxic chemicals. A method that could avoid all the above-mentioned limitations is the ideal choice for preparing AgNPs. Recently, it has been shown that several plant extracts can undergo highly controlled and hierarchical assembly, which makes them suitable for the development of a reliable and eco-friendly process (green synthesis) for metal nanoparticle synthesis(Geethalakshmi and Sarada, 2013; Vijayakumar et al., 2013; Jain et al., 2009; Vilchis-Nestor et al., 2008; Abu Bakar et al., 2007; Gregory et al., 2014c).

W. somnifera (L) Dunal, (solanaceae), commonly known as ashwagandha or winter cherry, is a well-known medicinal plant in Ayurvedic medicine. The principal active compounds include several withanolide-type compounds (Bhattacharya and Muruganandam, 2003; Mirjalili et al., 2009). Due to the nontoxic and high medicinal value of *W. somnifera*, this plant is widely used all over world. Roots, and less often leaves and fruits, have been used as phytomedicines in the form of decoction, infusions, ointment, powder and syrup (Bhattacharya and Muruganandam, 2003; Mirjalili et al., 2009; Gregory et al., 2014a). Nowadays, this plant is cultivated as a crop to support the high demand of biomass and a sustainable quality for pharmaceutical industry needs (Rajaswara Rao et al., 2012) . In this study, we biosynthesized AgNPs using *W. somnifera* extract and identified the compounds responsible for the formation of AgNPs. To demonstrate the potential pharmaceutical and industrial application of synthesized AgNPs, we have developed AgNPs- cream formulation and evaluated antimicrobial activity in a range of microorganisms.

5.3. Materials and Methods

5.3.1. Preparation of *W. somnifera* plant extract

W. somnifera (WS) plants were collected at Pondicherry Botanical Garden (Pondicherry, India). Leaves, fruits and roots were dried under shade at 25 °C and powdered. *W. somnifera* aqueous extracts (2, 4, 6 and 8 %, w/v) were prepared by boiling leaf, fruit and root biomass separately in 500 mL Erlenmeyer flasks containing 100 mL of sterile distilled water for 1 hrs. Aqueous extracts were obtained by centrifugation at 10,000 rpm for 10 min followed by filtration.

5.3.2. Synthesis of silver nanoparticles (AgNPs)

We screened several *W. somnifera* extracts for AgNPs formation. Firstly, 10 ml of 1mM AgNO3 solution was added to 500 µl aliquots of different concentrations (2, 4, 6 and 8 %, w/v) of roots, leaves and fruits aqueous *W. somnifera* extracts. The reaction occurred in 25 mL volumetric flasks at room temperature, solutions were vortexed (5min) thrice daily and continuously monitored for color changes as an indication of AgNO3 conversion into AgNPs. Simultaneously, aliquots were taken and analyzed for absorbance at 300–700 nm in a UV–Vis spectrophotometer. After AgNPs formation, the solution was centrifuged at 20,000 rpm for 15 min and supernatants discarded. Nanoparticles were washed with 10 ml of deionized water, centrifuged again, and supernatant discarded. Finally, AgNPs were re-dispersed in 10 ml of deionized water.

Since AgNPs were efficiently formed in 6% of aqueous leaf extract, we chose this extract for further standardization of AgNO3 concentration. Briefly, different concentrations of AgNO3 (0.1, 0.5, 1 and 2 m M) were prepared and 10 ml of each concentration were added to 6% w/v of leaf extract and the nanoparticle formation was recorded. As the combination of 6% leaf extract and 1mM AgNO3 found to be the optimum, AgNPs synthesized with this combination was used in all the experiments.

5.3.3. Physical characterization of AgNPs

5.3.3.1. Particle size and Zeta potential analysis

The size and zeta potential of AgNPs was determined by laser Doppler anemometry, using a Zetasizer (Zetasizernano ZS, Malvern Instruments, UK). Briefly, 100 µL of nanoparticle was diluted to 1 ml with deionized

water. An electric field of 150 mV was applied to observe the electrophoretic velocity of the particles (Marslin et al., 2009). All measurements were made at room temperature. Three independent samples run in triplicate were done.

5.3.3.2. Electron Microscopy analysis

Morphological characterization of the AgNPs was done by TEM and SEM analysis. For the TEM analyses the AgNPs were negatively stained with sodium uranyl acetate 1% solution and analysed using a transmission electron microscopy (TEM, JEOL-1400, 902A Jeol Ltd)(Gregory et al., 2014b). For the SEM analysis samples were prepared by dropping nanoparticle suspension onto aluminum stubs and allowing them to air-dry. The air-dried particles were sputter coated with gold under vacuum by using a Fiscon Instrument SC 502 and observed by SEM (Leica Cambridge S 360, Cambridge) for imaging. EDX analysis was conducted with the same instrument to confirm the elemental composition of the sample.

5.3.3.3. Atomic Force Microscopy (AFM)

The surface morphology of the AgNPs nanoparticles was studied by atomic force microscopy (AFM). A drop of the AgNPs was placed onto a 2x2 mm glass slide and left until the liquid had evaporated. Images were recorded using tapping mode in air on a Multi-mode Nanoscope IIa instrument equipped with a J scanner (Veeco Instruments, Santa Barbara, USA) and silicon nanoprobes (NCHV, Veeco). All images (600 nm wide) were fitted to a plane using the 1 degree flatten procedure included in the NanoScope software version 4.43r8.

5.3.3.4. X-ray diffractometry analysis

An x-ray diffraction (XRD) study of AgNPs was performed using an X-ray diffractometer (Philips, PW1710) with a horizontal goniometer. The samples were placed in the sample holder and scanned at a rate of 1° min^{-1} from 0° to 70°.

5.3.4. Chemical characterization of AgNPs

AgNPs and *W. somnifera* leaf aqueous extract were analysed by High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD) (Merck-Hitachi LaChrom Elite) (Dias et al., 1999) . Briefly, 2 mL of AgNPs were centrifuged and the pelleted AgNPs were extracted with 1 ml of 50% methanol. AgNPs extract solution and *W.*

somnifera leaf extract were filtered (Nylaflo membrane 0.45µM) prior to the HPLC analysis. Aliquots of these samples (20µL) were directly injected in HPLC. Chromatographic separations were carried out at 30 °C, on a LiChrospher RP-18e column (Merck, Germany), using 0.1% acetic acid in water (solution A), and 0.1% acetic acid in acetonitrile (solution B) as the mobile phases, at a flow rate of 0.7 ml/min. The following gradient was used: from 0 to 30 min, mobile phase A was decreased from 55% to 10% A. Data was recorded in the 200-520 nm range, and chromatograms recorded at 235 and 350 nm. Compounds were quantified by external standards of catechin, *p*-coumaric acid luteolin-7-glucoside (Sigma-Aldrich, Barcelona, Spain) and withanolides (Natural Remedies, India).

5.3.5. Antimicrobial activity studies

5.3.5.1. Screening of $AgNO_3$, W*. somnifera leaf extract and AgNPs for antimicrobial activity*

The preliminary antibacterial potential of AgNPs and the components used in the synthesis ($AgNO_3$ and *W. somnifera* leaf extract) was tested against *E. coli* by disc diffusion method. Briefly, 100 µl aliquots of *E. coli* culture were spread on petri dishes containing agar solidified LB medium. Sterile paper discs (5 mm diameter) impregnated with equivalent concentrations of AgNPs (20 µl), *W. somnifera* leaf extract and $AgNO_3$ solution were placed on the medium. Paper discs impregnated with water (20 µl) were used as control. The plates were incubated at 37 °C for 24 hrs and the inhibition zones were measured.

5.3.5.2. AgNPs cream preparation and its antimicrobial activity

Cream formulation with AgNPs was prepared with Croda base CR2 (EMFAL, MG, Brazil). Briefly, the CR2 was prepared at a concentration of 15% (w/v) in ultra-pure water (control) or containing 1% $AgNO_3$ ($AgNO_3$ cream) and equivalent AgNPs (AgNPs cream). The antimicrobial activities of the creams were evaluated by colony forming unit (CFU) method. Briefly, to 950 µl of grown microbial cultures (*S. aureus, P. aeruginosa, C. albicans, P. vulgaris, E. coli, A. tumefaciens*), 50 µl of cream ($AgNO_3$, AgNPs, or empty cream) at various dilutions were added and kept in 37 °C. Cultures added with 50 µl water served as positive control. After 24 hrs, 100 µl from each treatment and control were plated on LB agar medium using a sterile T- spreader. The colony forming units (CFUs) in each plate were counted after overnight incubation. All the experiments were done in quadruplicate.

5.4. Results and discussion

5.4.1. Green synthesis of silver nanoparticles

Among the different concentrations of *W. somnifera* extracts and $AgNO_3$ used for nanoparticles synthesis, 6 % w/v of WS and 1 mM $AgNO_3$ showed better reaction profiles and were selected for further studies. When $AgNO_3$ was added, leaf aqueous extract changed into a reddish color **Figure 1A**, whereas fruit and root extracts did not change their color within 7 days so withdrawn from studies. UV-Vis absorbance at 430 nm confirmed the reduction of silver ion to metal silver by the action of aqueous leaf extract of WS (Figure 1B). It was evident that WS leaf extract contains compounds suitable for green synthesis whereas roots and fruits extracts were not effective. Recently, the photosensitized synthesis of AgNPs via *W. somnifera* leaf powder was reported (Raut et al., 2014).

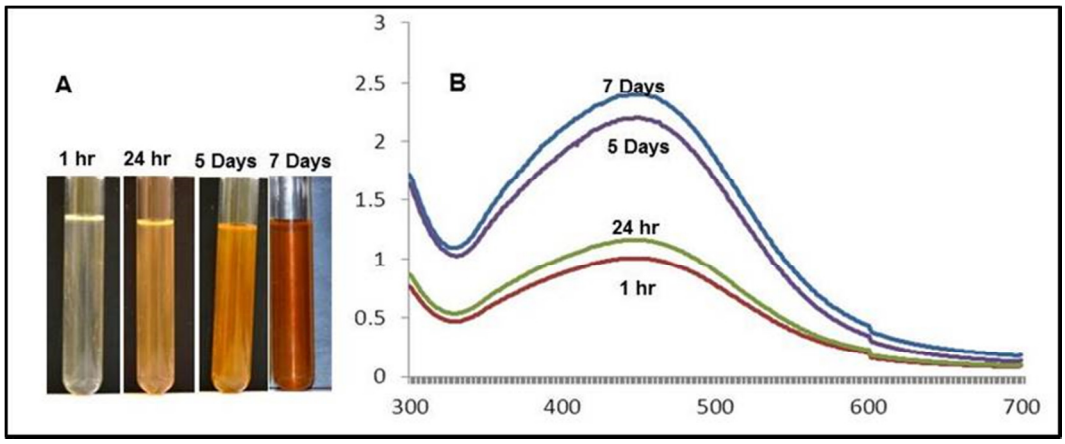

Figure 1. Photograph showing the AgNPs incorporated cream (A). UV–Vis spectra of AgNPs synthesized using 6% w/w *W. somnifera* aqueous extract with 1mM $AgNO_3$ at different time intervals (B), and visual observation of color changes.

5.4.2. Characterization of AgNPs

Particle size and size distribution are important characteristics of nanoparticles, as they determine their biological fate, toxicity, targeting ability and stability. The synthesized AgNPs had a size ranging between 70-110 nm, as confirmed by laser Doppler anemometry analysis (**Figure 2F**). The zeta potential was found to be -30.0±1.8 mv indicating the stability of the formulation.

The TEM, AFM and SEM images of AgNPs correlates with DLS data and showed particles with a spherical-irregular appearance (**Figure 2A-D**). Silver nanoparticles were brighter and easily identified by backscattered light in SEM

images, since metals like silver have higher secondary electron emission (**Figure 2C-D**). EDX spectrum of synthesized silver nanoparticles **Figure 2E** clearly exhibited the presence of elemental silver metal. The sharp signal peak of silver confirmed the reduction of silver nitrate to silver nanoparticles.

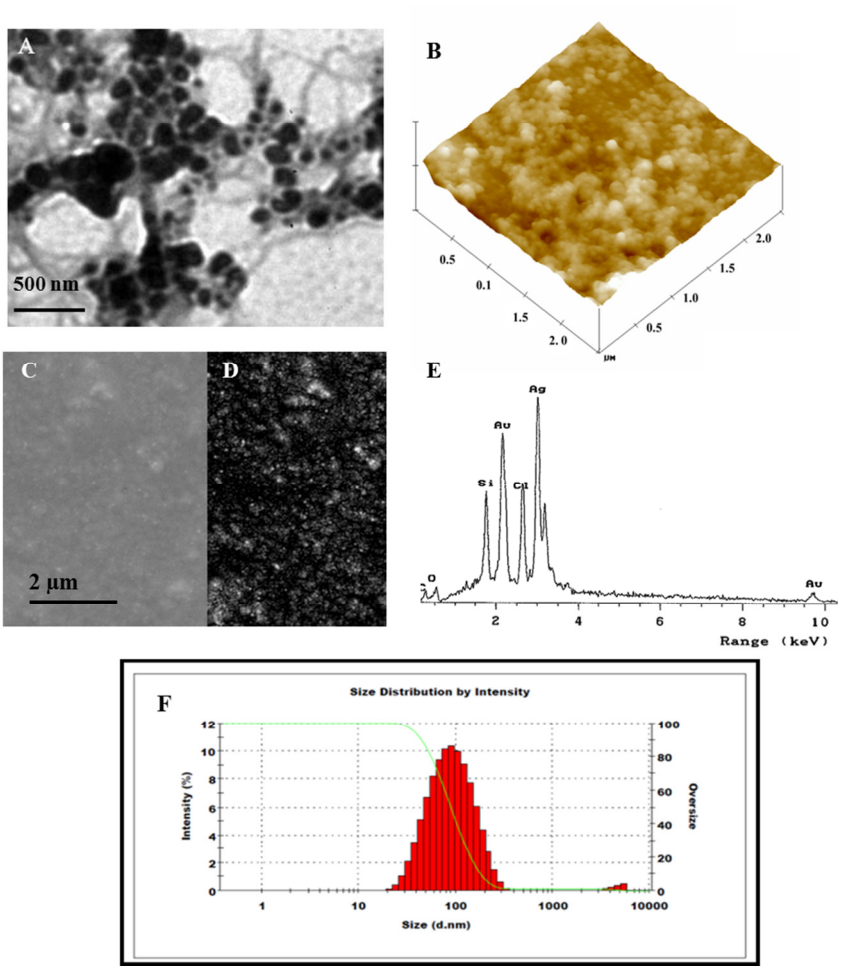

Figure 2. Morphological characterization of AgNPs. TEM images of (A), AFM height image (B) SEM primary image (C), Backscattered image (D), and X-ray energy dispersive spectrum (E) of AgNPs, (F) Particle size distribution of AgNPs.

The crystalline nature of AgNPs was confirmed by XRD analysis (**Figure 3**). The intensity of peaks reflects the high degree of crystallinity of the AgNPs. The distinct diffraction peaks at 38.06, 44.23 and 67.43 were indexed with planes (111) (200) (220). The well intense XRD pattern clearly showed the formation of AgNPs (JCPDS card no 04-0783, 1991) (Amutha et al. (2014) reported the same pattern of diffraction peak at 32.16 and indexed with planes (101), in biosynthesized AgNPs at room temperature. In the same way we got a peak at 32.20 and indexed with planes (101) (Amutha et al., 2014).

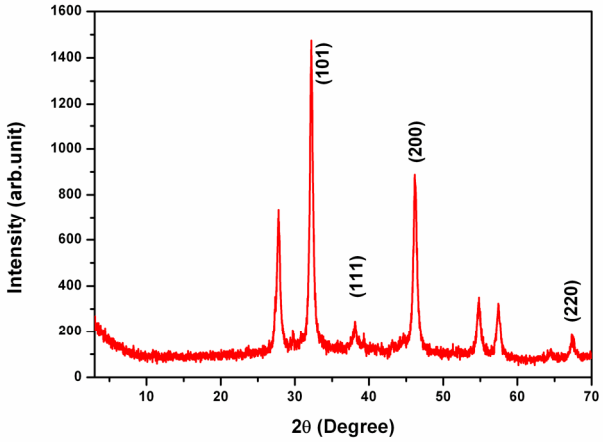

Figure 3. XRD patterns of the synthesized AgNPs with aqueous leaf extract of *W. somnifera.*

5.4.3. *W. somnifera* compounds responsible for green synthesis of AgNPs

Although green synthesis of AgNPs has been reported using extracts from different plant species, the knowledge concerning the phytocompounds responsible for green synthesis is still lacking. It was proposed that phenolics like catechin, may act as a reducing, stabilizing and capping agent (Tamuly et al., 2013; Nadagouda and Varma, 2008).

In order to identify the *W. somnifera* compounds participating in green synthesis of AgNPs, we performed HPLC analysis. We identified several withanolides in *W. somnifera* leaf extract based on their characteristic UV-Vis spectra (**Figure 4A-D; Table 1**). Additionally, HPLC analysis of AgNPs revealed that some *W. somnifera* phenolic compounds were selectively trapped in the nanoparticles (**Figure 4C, D**). These compounds, based on their characteristic UV-Vis spectra, and further confirmation with co-elution with pure standards, were identified as catechin, *p*-coumaric acid and luteolin-7-glucoside (**Figure 4C, D**, **and Table 1**). These substances were masked by other major compounds (peaks) present in *W. somnifera* extract, namely withanolides. In AgNPs chromatograms they were noticed since they were selectively trapped in AgNPs, being the other compounds removed when we washed the nanoparticles. Additionally, a major compound (peak 4) was found in AgNPs but not identified in the original *W. somnifera* leaf aqueous extract. This compound has UV-Vis spectra similar to other withanolides found in leaf aqueous extract (peaks W2, W5 and W6) but with a different retention time and showing a bathochromic effect on its spectra.

Table 1. Retention time of the compounds present in *W. somnifera* aqueous leaf extract and AgNPs.

Peak No	Retention time (min)		Compound
	Aqueous leaf extract	AgNPs	
W1	4.32	-	withanolide
W2	4.82	-	withanolide
W3	5.74	-	withanolide
W4	6.82	-	withanolide
W5	9.26	-	withanolide
W6	11.94	-	withanolide
1	-	2.98	catechin
2	-	3.29	*p*-coumaric acid
3	-	4.23	luteolin-7-glucoside
4	-	9.89	withanolide

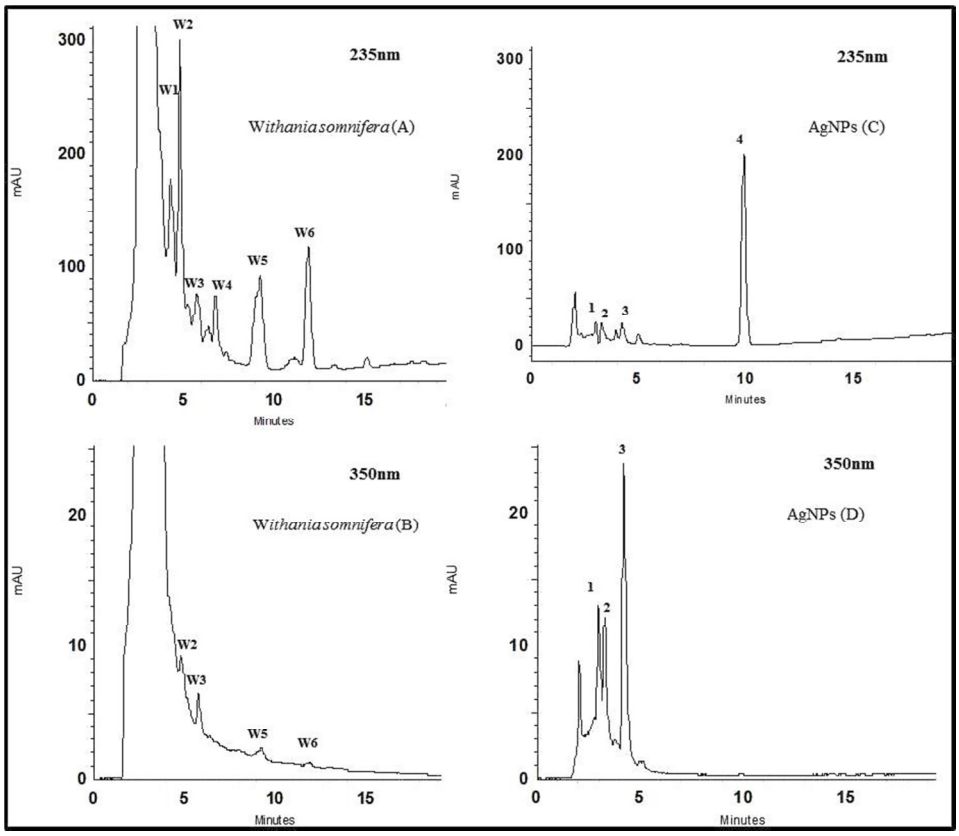

Figure 4. HPLC chromatograms of aqueous leaf extract of *W. somnifera* and green synthesized AgNPs. HPLC chromatogram of aqueous leaf extract of *W.somnifera* recorded at 235 and 350 nm (A and B). HPLC chromatogram of AgNPs recorded at 235 and 350 nm (C and D). Peaks identification is listed in Table 1.

Taking this in consideration, this compound might be a derivative originated from the interaction of some withanolide with silver ions. So we can conclude that the formation of AgNPs involved the interaction of silver ions

with both selected phenolics and withanolides present in *W. somnifera* aqueous leaf extract. Recently, the relevance of phenolic compounds for the green synthesis of metal nanoparticles from Eucalyptus globulus bark was reported (Santos et al., 2014).

5.4.4. Antimicrobial activity of AgNPs

It is well known that a number of chemical forms of silver exhibit antimicrobial activities (Lok et al., 2006). AgNPs showed a wider bacterial inhibition zone than $AgNO_3$ in all bacteria analysed (*E. coli, P. aeruginosa and A. tumefaciens*) (**Figure 5 A-C**).

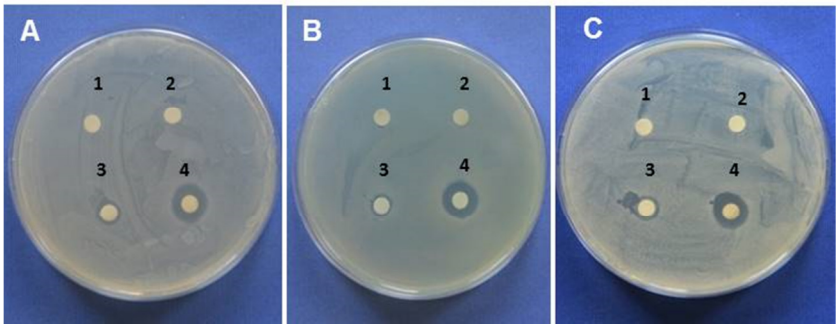

Figure 5. Anti-bacterial activity of AgNPs analyzed by disc diffusion method. Bacterial growth without treatments (1) or treated with equivalent amount of *W. somnifera* aqueous extract (2), $AgNO_3$ (3) and AgNPs (4).The bacteria tested were *E.coli* (A), *P. aeruginosa*(B), and *A. tumefaciens* (C).

AgNPs interaction with *E.coli* cells were studied using SEM analysis (**Figure 6**). The AgNPs treated cells (2 hrs and 4 hrs) showed increased disruption in the cell wall of *E. coli* with increase time interval.

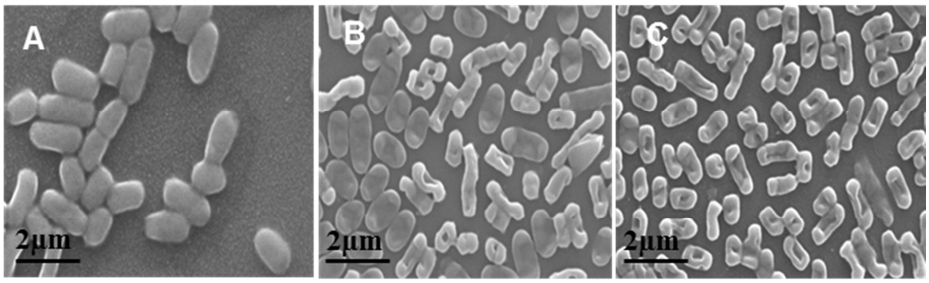

Figure 6. SEM analysis of AgNPs and *E. coli* interaction. Cellular interaction of AgNPs and *E.coli* visualized by scanning electron microscopy (SEM) analysis: control (A), after 2 hrs (B) and 4 hrs (C) incubation time.

No disruption was seen in untreated cells, clearly indicating the enhanced antibacterial activity of AgNPs (**Figure 6 A-C**).

The results from colony counting method showed that AgNPs significantly reduced the number of *E.coli* cells from 10^6 to 1.3±0.9 CFUs, while the reduction with $AgNO_3$ treatment was 258.6±9.9, and no reduction was observed with *W. somnifera* aqueous extract treatment (**Figure 7**). These results further substantiate the higher antimicrobial activity (aprox. 200 times more effective) of AgNPs comparing to equivalent amounts of $AgNO_3$. It is well reported that silver nanoparticles have a higher activity then $AgNO_3$ counterparts (Velusamy et al., 2015). Moreover, plant extract biosynthesized silver nanoparticles showed enhanced antibacterial activity compared to chemically synthesize silver nanoparticles (Mukherjee et al., 2014).

The lethality of AgNPs can occur by physical process such as damaging bacterial cellular proteins, disruption of cell membrane, blocking the microbial respiratory chain system and penetration of nanoparticles in to the cytoplasm(Dror-Ehre et al., 2009; Zhang et al., 2008). The higher antibacterial activity of AgNPs, compared with $AgNO_3$, could also be due to the presence of *W. somnifera* phenolics in the nanoparticles. It is well known that some phenolics can have antimicrobial activities or potentiate the activity of antibiotics, increasing their efficacy.

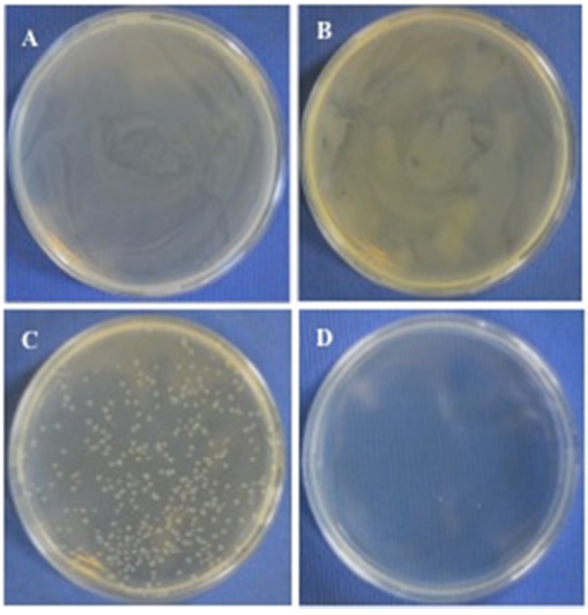

Figure 7. Anti-bacterial potential of AgNPs by colony counting method. *E.coli* without treatment (just water) (A), *E.coli* treated with plant extract (B), *E.coli* treated with $AgNO_3$ (c), and *E.coli* treated with AgNPs (D).

Compounds like flavones and cathecin derivatives, structurally similar to the ones identified in *W. somnifera* and AgNPs, have been shown to have light antimicrobial activity (Cushnie and Lamb, 2011; Daglia, 2012). Several tea catechins in combination with oxacillin (ß-lactam antibiotic) increase its activity against several strains of *S. aureus* up to 128-fold (Daglia, 2012). Some antimicrobial mechanisms of the phenolics are related with efflux pump inactivation and cytoplasmic membrane destabilization(Cushnie and Lamb, 2011), which might explain the observed effects (SEM analysis) of AgNPs in bacterial cells **Figure 6** and their higher activity compared with $AgNO_3$ or *W. somnifera* extract alone (**Figure 7**).

Antibiotic creams are used to speed the healing of wounds and prevent infection. Antibacterial activity of creams incorporated with $AgNO_3$ and AgNPs were compared with an empty cream, against pathogenic clinical microbial isolates of *S. aureus, P. aeruginosa, C.albicans, P. vulgarius, E. coli,* and the plant pathogen *A. tumefaciens.* The results showed that AgNPs cream significantly reduced the number of colonies against all the tested pathogens compared to the cream containing $AgNO_3$ (**Table 2**). These results further substantiate the higher antimicrobial activity against the Gram-positive bacteria *S. aureus* (aprox. 200 times more effective) of AgNPs-cream comparing to $AgNO_3$-cream. Similarly, Gram-negative bacteria's *viz, P. aeruginosa, P. vulgarius, E. coli,* and *A. tumefaciens* also showed higher reduction of CFU when treated with AgNPs (31 times, 43 times, 28 times and 13 times more, respectively, compared to $AgNO_3$). AgNPs also showed 20 time greater anti- microbial activity compared with $AgNO_3$ in fungal species *C. albicans*. These results pointed-out AgNPs has higher antimicrobial potential against both Gram–positive and Gram-negative, and fungal pathogens.

Silver-based products with antimicrobial properties are employed in different industries like medical (e.g. antibacterial creams), food (e.g. antibacterial agents in packaging) and in textile (e.g. preparation of silver wools),(Rai et al., 2009). The incorporation of silver as nanoparticles has been envisaged as an alternative solution, making those industries more environmental safe. The biosynthesised *W. somnifera* AgNPs and *W. somnifera* AgNPs-cream showed to have a strong antimicrobial activity, higher than the AgNO3 counterparts (**Figure 7 and Table 2**). Silver formulations (gels, creams, etc.) are widely used in health sector namely in wound care.

Table 2: Evaluation of antimicrobial potential of AgNPs incorporated cream.

Organisms	Water (Control) (CFU)	Empty Cream (CFU)	$AgNO_3$ in Cream (CFU)	AgNPs in cream (CFU)

Stapylococcus aureus	226.3 ± 0.74	211.0± 0.69*	123.3± 0.74***	0.6± .25$^{***,+++}$
Pseudomonas aeruginosa	432.0 ± 0.69	408.0± 1.00*	267.6± 0.67***	8.6± .48$^{***,+++}$
Candida albicans	264.6± 0.88	127.6±0.75***	33.3±0.68***	1.6±0.25$^{***,+++}$
Proteus vulgaris	213.0± 1.17	194.6 ± 1.02	26 .6±0.64***	0.6±0.25$^{***,+++}$
Escherichia coli	471.0± 1.39	441.3±1.36	226.0±1.16***	8.0±0.66$^{***,+++}$
Agrobacterium tumefaciens	190.3±1.47	145.3±0.88*	35.0±1.02***	2.66±0.58$^{***,++}$

Data were analyzed by non-parametric T-test using Graph Pad Prism5 software (GraphPad, USA). Data were presented as mean ± SEM of 4 replications. Asterisks (* P<0.05, ** P<0.01, ***P<0.001) denotes statistically significant different from water control. Asterisks (+) denote statistically significant of AgNPs ($^{+}$ P<0.05, $^{++}$ P<0.01, $^{+++}$P<0.001) compared with different $AgNO_3$.

The AgNPs-cream here produced might be used for the same purposes with advantage, since it is more effective (antimicrobial) and so can be used in lower doses, with less toxicity for the patient and being more eco-friendly.

5.5. Conclusion

In conclusion, *W. somnifera* aqueous leaf extracts are suitable for the green synthesis of AgNPs with potent antimicrobial activity. Moreover, it was found that catechin, *p*-couparic acid, luteolin-7-glucoside and a non-identified withanolide are compounds present in *W. somnifera* aqueous leaf extract responsible for green synthesis of AgNPs. The antimicrobial study concludes that AgNPs are 200 times more potent when compared to $AgNO_3$. The AgNPs disrupt the cell membrane of *E. coli* as confirmed by SEM analysis. This AgNPs and cream incorporated AgNPs has a potential application in many different industries including medical, food and textile.

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Chapter 6:
Conclusions

6. Conclusions

During the recent years, extensive research is going on in the area of nano drug delivery of commercial drugs and plant bioactive compounds. However, research in the area of nanoencapsulation of extracts for targeted drug delivery is still at the early stage. More research should be focused on the carrier materials in order to develop suitable carriers, which can enhance their activity and improve the overall efficacy of plant extracts. Through nanotechnology, value added drug delivery systems could be developed to avail the complete therapeutic potentials of the herbal drugs. Thus, herbal medicine packed into polymers as nanoparticles is a successful way to overcome the draw backs of conventional therapies. For example poor aqueous solubility and permeability are two main limitations of phytomedicines, severely affecting their efficacy. In the present investigation (i) we have developed HPLC method for the quantification of bioactive compounds from extracts obtained from different parts *W. somnifera* and to quantify curcumin from *C. longa* and *C. aromatica.* (ii*) W. somnifera* extract and curcumin were encapsulated in PCL and MPEG-PCL nanoparticles. (iii) *W. somnifera* extract and curcumin loaded nanoparticles were characterized for their size, shape, encapsulation efficiency and drug release profile (iv) Characterized nanoparticles in comparison with free extract or compound were tested for their *in vitro* cytotoxicity, cellular uptake and *in vitro* neuroprotection in U251 glioma cells. *In vivo* bio localization of nanoparticles was analyzed in Zebrafish model. The major conclusions of the present thesis research are summarized below.

HPLC method developed for the quantification of withanolides is new, simple, fast and reproducible. This method would enable the simultaneous estimation of withanolides withanolide-A, withanolide-B, withanone, withaferin-A and 12-deoxy-withastramonolide. We have also found that 90% methanolic extract obtained from leaf tissues possessed the highest amount of withanolides. Purospher. RP-18 5µm column is ideal for efficient separation of compounds. Regarding curcumin, we report that *C. longa* species has higher amount of curcumin compared to *C. aromatica*.

Although herbal extracts possess excellent *in vitro* bioactivity, their poor aqueous solubility, larger molecular size and extensive metabolism limits their clinical outcome. Application of nanotechnology increases the bioavailability and bioactivity of phytomedicines by modifying their surface and reducing the size of the particles. By enabling targeted and sustained delivery of the drug, nanotechnology improves the pharmacokinetics profile and drug diffusion in various organs including crossing the BBB. In the present study, *W. somnifera* extract and curcumin

were encapsulated in PCL and MPEG-PCL nanoparticles by solvent displacement method. The sizes of the nanoparticles were influenced by polymer to drug ratio. The prepared nanoparticles were spherical in shape with a smooth surface. Importantly, nanoencapsulation greatly improved the aqueous solubility of both *W. somnifera* extract and curcumin.

Irrespective of the loaded materials (*W. somnifera* and curcumin), the polymer used for encapsulation (PCL and MPEG-PCL) played an important role. *In vitro* and *in vivo* cellular uptake and neuroprotective effect *W. somnifera* extract and curcumin loaded MPEG-PCL nanoparticles was higher compared to their PCL formulations and free forms. Accordingly, both *W. somnifera* extract and curcumin loaded MPEG-PCL nanoparticles showed higher protective effect against oxidative damage induced by tBHP in U251 glioma cells. Together, our results suggest that MPEG-PCL nano encapsulation of *W. somnifera* extract /curcumin enhances their aqueous solubility and cellular uptake. We believe that these formulations can be used as potential candidates for protecting the neurons from oxidative damage.

Our study has also revealed that *W. somnifera* aqueous leaf extracts are suitable for green synthesis of silver nanoparticles with potent antimicrobial activity. Although there are several studies on the green synthesis of silver nanoparticles, the compounds responsible for this synthesis are not well known. In this context, we found that catechin, *p*-couparic acid, luteolin-7-glucoside and a non-identified withanolide are the compounds present in *W. somnifera* aqueous leaf extract are responsible for green synthesis of silver nanoparticles. From the antimicrobial activity assay, we could conclude that silver nanoparticles synthesized using *W. somnifera* extract is 200 times more effective compared to $AgNO_3$. This silver nanoparticle cream formulation developed in this study would have potential applications in medical, food and textile industries.

6.4 References

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