URO PROTECTIVE EFFECT OF LUTEOLIN NANO-SUSPENSION ON CYCLOPHOSPHAMIDE-INDUCED HEMORRHAGIC CYSTITIS IN RATS

RAVINDRAN PILLAI HARIKUMAR

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the degree of Doctor of Philosophy

June 2016

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DEDICATIONS

This thesis is dedicated to

All my teachers (guru) for their benevolence, care, inspiration and devotion

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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June 2016

Chairman : Rajesh Ramasamy, PhD
Faculty : Medicine and Health Sciences

Plant-derived extracts have been a great source of materials utilized in medical treatment since immemorial time. According to the World Health Organization (WHO), 70-95 % of human population depends on traditional medicines for their healthcare in which herbal drugs are commonly employed. Luteolin is a bioflavonoid that known for its anti-inflammatory, anti-diabetic, anti-cancer and chemoprotective properties nevertheless it relatively possesses low water solubility and oral bioavailability. The present study was aimed to develop luteolin nanosuspension (LNS) with increased oral bioavailability and to evaluate its protective effect on cyclophosphamide (CP) induced hemorrhagic cystitis (HC) in SD rats.

High-pressure homogenization (HPH) technique was used to prepare LNS. LNS showed an enhanced aqueous solubility, which may be attributed to the reduction of particle size from 1649.3 ± 432.23 nm to 420.4 ± 11.26 nm. The oral bioavailability of LNS was determined using HPLC-UV technique, where LNS (30 mg/kg/body weight) and non-modified pure luteolin (30 mg/kg/body weight) were given to rats. LNS significantly improved the oral bioavailability (54.41±14.49%) which was 1.5 fold higher (p<0.05) than pure luteolin (21.72±3.89%).

The uroprotective function of LNS was assessed in CP-induced acute hemorrhagic cystitis rats. Male SD rats were divided into four groups. Group-1 (vehicle control) received normal saline (10 ml/kg/b.w/p.o); group-2 (negative control) received three doses of CP (100 mg/kg/b.w/p.o) on day 6, 8, 10; group-3 (positive control) received MESNA (20 mg/kg/b.w/p.o) and group-4 (test) received LNS (30 mg/kg/b.w/p.o). The relative concentration of glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrite and leukocyte in urine were detected using urine strip test to indicate

the incidence the severity of HC. Urinary bladder of CP treated animals reflected severe hemorrhage, inflammation, edema and deep red colouration whereas; LNS significantly improved the morphological deterioration perpetrated by CP.

CP treatment has significantly reduced the expression levels of SOD, CAT, GPx, GADD45A and increased the expression levels of TNF-α, *p53*, NFkB and VEGF. However, LNS administration along with CP have protected the urinary bladder from HC conditions by increasing the expression levels of antioxidant enzymes SOD, CAT and GPx and also by inhibiting the expressions of genes that responsible for bladder inflammation including TNF-α , NFkB and VEGF. Moreover, LNS treatment significantly elevated the serum levels of cytokines IL-2 and IL-4, which may reduce the CP-induced bladder hemorrhage, inflammation and associated symptoms. Besides, the hepatic enzyme level of AST, ALT, ALP, LDH, total protein and creatinine were normalized in LNS treatment group. The histological examination demonstrated that uroepithelial tissue damages were also prevented by the LNS in CP-induced hemorrhagic cystitis pathogenesis.

In conclusion, HPH technique could serve as an ideal, easy, safe and reproducible technique to formulate LNS. LNS protected the urinary bladder from CP-induced toxicity by modulating the release of various inflammatory mediators; enhancing the immune status of the host; restoring the oxidative/redox mechanisms and effectively clearing the toxic metabolite residues in the urinary bladder. LNS with its anti-oxidative, anti-inflammatory and chemoprotective activities had alleviated the HC conditions induced by CP. The current study supports the use of luteolin in nanosuspension form (LNS) as uroprotectant along with drugs that pathologically affect urinary bladder.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN UROPROTEKTIF OLEH NANOSUSPENSI LUTEOLIN KEATAS TIKUS SISTITIS BERDARAH DIARUH OLEH CYCLOPHOSPHAMIDE

Oleh

RAVINDRAN PILLAI HARIKUMAR

Jun 2016

Pengerusi : Rajesh Ramasamy, PhD
Fakulti : Perubatan dan Sains Kesihatan

Ekstrak tumbuh-tumbuhan adalah salah satu sumber yang digunakan dalam rawatan perubatan sejak zaman dahulu lagi. Menurut Pertubuhan Kesihatan Sedunia (WHO), seramai 70-95% penduduk dunia bergantung kepada ubat-ubatan tradisional yang diterbi dari herba untuk penjagaan kesihatan. Antara ekstrak tumbuh-tumbuhan, luteolin merupakan bioflavonoid yang terkenal dengan aktiviti-aktiviti anti-inflamasi, anti-diabetes, anti-kanser dan kimoprotektif. Namun, luteolin mempunyai keterlarutan air dan bioavalabiliti yang rendah. Oleh hal yang demikian, kajian ini bertujuan untuk membangunkan sebatian nanosuspensi luteolin (LNS) dengan tahap bioavailability yang tinggi serta menilai kesan perlindunganya terhadap sistitis berdarah (HC) yang diaruh oleh ‘cyclophosphamide’ (CP) pada tikus SD.

Teknik ‘high pressure homogenizer’ (HPH) telah digunakan untuk menyedia LNS. LNS yang terhasil menunjukkan kelarutan air yang tinggi serta saiz molekul yang kecil yang dikurangkan daripada 1649.3 ± 432.23 nm sehingga 420.4 ± 11.26 nm. Bioavailabiliti oral LNS ditentukan melalui teknik HPLC-UV, dimana LNS (30 mg/kg/berat badan) dan luteolin tulen (30mg/kg/berat badan) diberi secara oral kepada tikus. Didapati bahawa, bioavailabiliti oral luteolin ditingkatkan apabila diberi dalam bentuk LNS (54.41± 14.49%) iaitu 2.5 kali ganda lebih tinggi ($p<0.05$) daripada luteolin tulen (21.72± 3.89%).
Fungsi uroprotektif LNS dinilai dengan menggunakan model tikus HC yang diaruh oleh CP. Tikus SD jantan telah dibahagikan kepada empat kumpulan. Kumpulan-1 (kawalan ‘vehicle’) menerima air ‘saline’ (10ml/kg/bw/p.o); kumpulan-2 (kawalan negatif) diberi tiga dos CP (100 mg/kg /bw/p.o) pada hari ke-6, 8, 10; kumpulan 3 (kawalan positif) menerima MESNA (20mg/kg/bw /p.o), manakala kumpulan-4 (ujian) dirawat dengan LNS (30mg/kg/bw/p.o). Kandungan glukosa, bilirubin, ketone, pH, protein, urobilinogen, nitrit dan leukosit dalam air kencing ditentukan untuk menggaris darjah keterukan HC. Didapati bahawa, pundi kencing haiwan

diberi CP (kawalan) mengalami pendarahan yang teruk disertai dengan inflamasi and edema. Manakala, kumpulan haiwan yang dirawat dengan LNS dicegah daripada mengalami perubahan morfologi ketara akibat daripada ketoksikan CP.

Rawatan CP juga turut merendahkan ekspresi SOD, CAT, GPx, GADD45A dan meningkatkan tahap ekspresi TNF-α, *p*53, NFkB dan VEGF. Akan tetapi, rawatan CP bersama dengan LNS menunjukan fungsi perlindungan pundi kencing dimana tahap ekspresi enzim-enzim antioksidan SOD, CAT dan GPx ditingkatkan dan ekspresi gen yang bertanggungjawab untuk inflamasi seperti TNF-α, NFkB dan VEGF telah dikurangkan. Rawatan LNS juga meningkatkan paras sitokin IL-2 dan IL-4 di serum yang mampu mengurangkan gejala-gejala negatif yang diaruhkan oleh CP. Selain itu, LNS juga menonjolkan fungsi hepatoprotektif dengan menormalkan paras enzim-enzim hepatik, AST, ALT, ALP, LDH, jumlah protein dan kreatinin. Pemeriksaan histologi selanjutnya membuktikan bahawa kerosakan tisu urotelia turut dicegah oleh LNS apabila diberi bersama CP.

Kesimpulannya, teknik HPH adalah ideal, mudah, selamat dan ‘reproducable’ untuk membangunkan LNS. LNS melindungi pundi kencing daripada ketoksikan CP menerusi modulasi perembesan pelbagai pengantara inflamasi; meningkatkan status imun; membetulkan mekanisma oksidatif/redoks dan membersihkan metabolit toksik yang terkumpul di dalam pundi kencing. Sebatian LNS dengan fingsi anti-oksida, anti-inflamasi dan kimoprotektif menggurangkan fenomena HC yang diaruh oleh CP. Jesturu, kajian ini menyokong penggunaan luteolin dalam bentuk nanosuspensi (LNS) sebagai agen uroprotektif apabila diguna bersama dengan ubat-ubatan yang menjejaskan pundi kencing.

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I certify that a Thesis Examination Committee has met on 9 June 2016 to conduct the final examination of Ravindran Pillai Harikumar on his thesis entitled "URO Protective Effect of Luteolin Nano Suspension on Cyclophosphamide-Induced Hemorrhagic Cystitis in Rats" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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(Internal Examiner)

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Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Internal Examiner)

Syam Mohan Murali Mohan, PhD
Associate Professor
Jazan University
Saudi Arabia
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 23 August 2016

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of Supervisory Committee were as follows:

Rajesh Ramasamy, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

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Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Aishah Adam, B Pharm, PhD
Professor
Faculty of Pharmacy
Universiti Teknologi MARA (UiTM)
(Member)

Dharmalingam Senthil Rajan, PhD
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Faculty of Pharmacy
International Medical University
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Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Khozirah Shaari

Signature: _____
Name of Member of Supervisory Committee: Professor Dr. Aishah Adam, B Pharm

Signature: _____
Name of Member of Supervisory Committee: Dr. Dharmalingam Senthil Rajan

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LIST OF ABBREVATIONS

AUC	Area under curve
AUMC	Area under moment curve
CAT	Catalase
C_{max}	Concentration maximum
COX-2	Cyclooxygenase-2
CP	Cyclophosphamide
DLS	Dynamic light scattering
GADD45A	Growth arrest and DNA damage inducible alpha
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H HC	Hemorrhagic cystitis
H_A	Alternative hypothesis
H_o	Null hypothesis
HPH	High pressure homogenization
ICAM	Intercellular adhesion molecule
K_{el}	Elimination constant
LD	Laser diffraction
LDH	Lactate dehydrogenase
LNS	Luteolin nanosuspension
LPS	Lipopolysacchride
MAPK	Mitogen activated protein kinase
MESNA	Mercapto ethane sulfonate sodium
MRT	Mean residence time
NFkB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
p53	Tumor protein *p53*
PAM	Phosphoramide mustard
PCS	Photon correlation spectroscopy
PDI	Poly dispersity index
PK	Pharmacokinetics
PSI	Pound per square inch

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
$T_{1/2}$	Half life
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
ZP	Zeta potential

CHAPTER 1

INTRODUCTION

Humans consume food and herbal medicines since they have started living on this planet. They lead by instinct, guided through experience, and latterly, by logical thinking (Havsteen, 2002). Plant-derived compounds have been a great source of materials used in beneficial medical treatment since the time of immemorial. As per WHO assessment, 70-95% of universal community employs traditional, herbal drugs for the healthcare (Robinson and Zhang, 2011). Epidemiological researches have established a link between higher fruit and vegetable intake and reduced incidence of human diseases (Wang *et al.*, 2014), but failed to decipher the molecules responsible, or resulted due to the synergistic effects of the natural food.

Dietary materials consist of fruits and vegetables have an ability to protect an array of ailments, especially cardiovascular system (CVS) disorders and malignant syndrome (Ness and Powles, 1997). Anti-oxidants and natural fibers constitute the major supplements liable for these protective properties. Reactive oxygen species (ROS) developed due to metabolism and capable of damaging macromolecules like DNA, proteins, and lipids. The aggregation of these molecules eventually lead to the occurrence of chronic ailments including cancer, diabetes and inflammatory diseases (Halliwell, 1994). Among all other herbal components, flavonoids are common constituents of plants used in traditional medicine to treat a wide range of diseases. Many researches carried out analysis regarding the absorption, bioavailability and tissue distribution pattern of flavonoid compounds (Hollman and Katan, 1999). Dietary absorption pattern of flavonoids was considered to be low since it was linked with glycosides.

Luteolin is a bio-flavonoid present in natural foods and in traditional medicinal plants with profound pharmacological properties (Lopez, 2009). Luteolin can also combine with other anti-oxidants like vitamins. Luteolin at low concentrations exhibit anti-inflammatory properties, though explained partly by its anti-oxidant effects. Luteolin exhibits good radical scavenging and chemoprotective properties (Kumar and Pandey, 2013). Luteolin exhibit numerous pharmacological properties, including anti-oxidant, anti-inflammatory and anti-microbial functions (Lopez, 2009). Only limited data on the oral bioavailability of luteolin is available; thus more quantitative research is required to solicit the pharmacological competence of luteolin (Seelinger *et al.*, 2008).

Many new compounds have low aqueous solubility, and hence with low oral bioavailability (Bansal *et al.*, 2011). The efficacies of any pharmaceutical or dietary products in modulating diseases rely on conserving the bioavailability of the active principles. The delivery of phytochemicals hence needed

adequate formulations to maintain their bioactive potential, absorption, tissue distribution and stability parameters. Nanoparticle formulation technology has all the above characteristics and well appreciated by scientific community.

Nanotechnology based formulation aspects has been employed in pharmaceutical industry for solubilisation and to improve dissolution of poorly soluble molecules. Comprehensive research interest has been established in nanoparticle drug delivery technologies. Unlike to other nano-carriers, the nanosuspension contains no carriers, but a nanosized dispersion of poorly soluble molecules in crystalline state (Müller *et al.*, 2001) or amorphous state (Chingunpituk, 2011), preserved by surface active agents. If the molecules remain in the crystalline state, such nanosuspension may be known as nanocrystals (Junghanns and Müller, 2008). Numerous preparation technologies have been established for nanosuspension on the basis of two approaches, wet milling (Liversidge and Cundy, 1995) and high pressure homogenization (HPH) (Keck and Müller, 2006; Müller and Peters, 1998).

Muller and his group in early 90's have added to an option innovation in light of piston gap high pressure homogenization to deliver nanoparticulate suspensions, called Disso-Cubes™. Indicated by the cubic state of the medication nanocrystals delivered with this procedure (Muller *et al.*, 2002). This innovation is a regular top-down procedure which depends on jet stream homogenization. The medication is pumped under high pressure of up to 1700 bar through a micro scale fluidizer framework (Junghanns and Müller, 2008). HPH system utilizes collision chamber of either Z-type or Y-type, during particle collision, shear forces and cavitations forces leading to the required particle size reduction. The subsequent molecule sizes are saved by the utilization of different phospholipids or different surfactants and stabilizers.

High pressure homogenization (HPH) is the technique used to create luteolin nanosuspension (LNS) in which, particle diminution to the submicron extent used to build the dissolution rate, the saturation solubility and thus to improve the oral bioavailability of inadequately dissolvable drugs (Merisko and Liversidge, 2011).

The nanosuspensions which contain luteolin were prepared by HPH using Avestin high pressure homogenizer. Generally nanoparticle-drug formulations enhance drug potency, specificity, tolerability and therapeutic index of respective molecules. Moreover, nanosuspensions can also be produced cost effectively and with high drug loading capacity that ultimately reduces doses of application (Patel and Agrawal, 2011).

Pharmacokinetics (PK) is a branch of science deals with drug absorbed from different routes, distribution to tissues and organs, metabolism, and

excretion. PK determines the fate of the compounds from intake until they are completely excreted from the body. PK and tissue distribution analysis helps researchers to design and develop formulations of a particular drug. By the optimal use of drug formulation, the drug release to the proper tissues/organs can be regulated and minimize the drug circulation to the non-target tissues. Thus, results in improved pharmacological properties with low untoward actions (Li and Huang, 2008).

1.1 Problem statement

- Cyclophosphamide (CP) one of the commonly used oxazaphosphorine has been chosen as a model compound in many scientific experiments (Dobrek and Thor, 2012). Major side effects of CP include the urological disturbances, like voiding symptoms, suprapubic discomfort and to dangerous haemorrhagic cystitis (HC).
- The simultaneous administration of sodium-2-mercaptoethane (Mesna) is the most widely employed methods for prevention of CP-induced HC in clinical practice. However, anaphylactic shocks, allergic symptoms including urticaria, skin rashes, hematuria and elevated levels hepatic enzymes are the major side effects of Mesna treatment (Manikandan *et al.*, 2010). It was estimated that HC also occurs in 10-40% of Mesna-treated patients (Brock and Pohl, 1983). Therefore, there is a need for new effective agents for prevention of this side effect.
- Natural compounds or herbal extracts containing flavonoids have been investigated as modulators of CP induced urotoxicity. Thus the current study was aimed to explore the role of luteolin in exerting uroprotective activity in experimental model and deciphering the underlying the mechanism/s. Since luteolin has a low oral bioavailability (Chen *et al.*, 2012) it has been converted to luteolin nanosuspension form.
- Although limited data for oral absorption and metabolism are available, a quantitative study is essential to ascertain the pharmacological activities of luteolin (Seelinger *et al.*, 2008). As oral administration is the most accepted method of administration of drugs, luteolin may be formulated as an oral nanosuspension form and it is essential to identify the bioavailability pattern, distribution and elimination of luteolin after oral administration (Chen *et al.*, 2012). To successfully use the various pharmacological properties of the bioflavonoid luteolin and to bypass its low oral absorption, there is a need to design a novel formulation that can enhance oral absorption.

1.2 Research hypothesis

- H_0: Luteolin nanosuspension will not improve the oral bioavailability of luteolin to elicit uroprotective effect compared to pure luteolin in male SD rats.
- H_A: Luteolin nanosuspension will improve the oral bioavailability of luteolin to elicit uroprotective effect compared with pure luteolin in male SD rats.

1.3 General objective

- To determine the uroprotective function of luteolin nanosuspension on cyclophosphamide-induced hemorrhagic cystitis in rats.

1.4 Specific objectives

- To prepare and evaluate the morphological, physico-chemical, stability properties of luteolin nanosuspension (LNS).
- To evaluate acute oral toxicity of LNS.
- To determine the relative bioavailability and pharmacokinetics of luteolin in SD rats.
- To evaluate the protective effect of LNS on hemorrhagic cystitis in murine models.
- To elucidate the potential molecular mechanisms of the uroprotective effect of LNS in murine urinary bladder.

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APPENDICES

Appendix-1

CLINICAL OBSERVATION RECORD-

Acute Toxicity Study

Name of Investigator: Harikumar Test Item: Luteolin Dose: 2ml/100g Strain: Female SD rats

Date: **10-01-2014** to **24-01-2014** Animal ID: 1 Time of Dosing: 9am

Clinical observations (Caged)	**0 Day**				**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	30 min	**1h**	**2h**	**4h**														
Mortality	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Moribund	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Convulsions	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Tremor	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Straubs Tail	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Locomotor Activity	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Abnormal Gait	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Streotypic Reaction	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Piloerection	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Writhing	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Tachypnea	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Dyspnea	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Urination	✓	×	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Feces	×	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Alopecia	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Bleeding	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×

Appendix-2

CLINICAL OBSERVATION RECORD

Name of Investigator: Harikumar Test Item: Luteolin Dose: 2ml/100g Strain: SD rats

Date: 10-01-2014 to 24-01-2014 Animal ID: 1 Time of Dosing: 9.30am

Clinical observations	0 Day				1	2	3	4	5	6	7	8	9	10	11	12	13	14
	30 min	1h	2h	4h														
Hand Held Observations																		
Vocalization	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Nasal Discharge	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Salivation	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Lacrimation	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Exopthalmos	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Ptosis	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Loss of Corneal Reflex	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Loss of Righting Reflex	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Loss of Grasping reflex	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Diarrhea	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Analgesia	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Others	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Signature																		

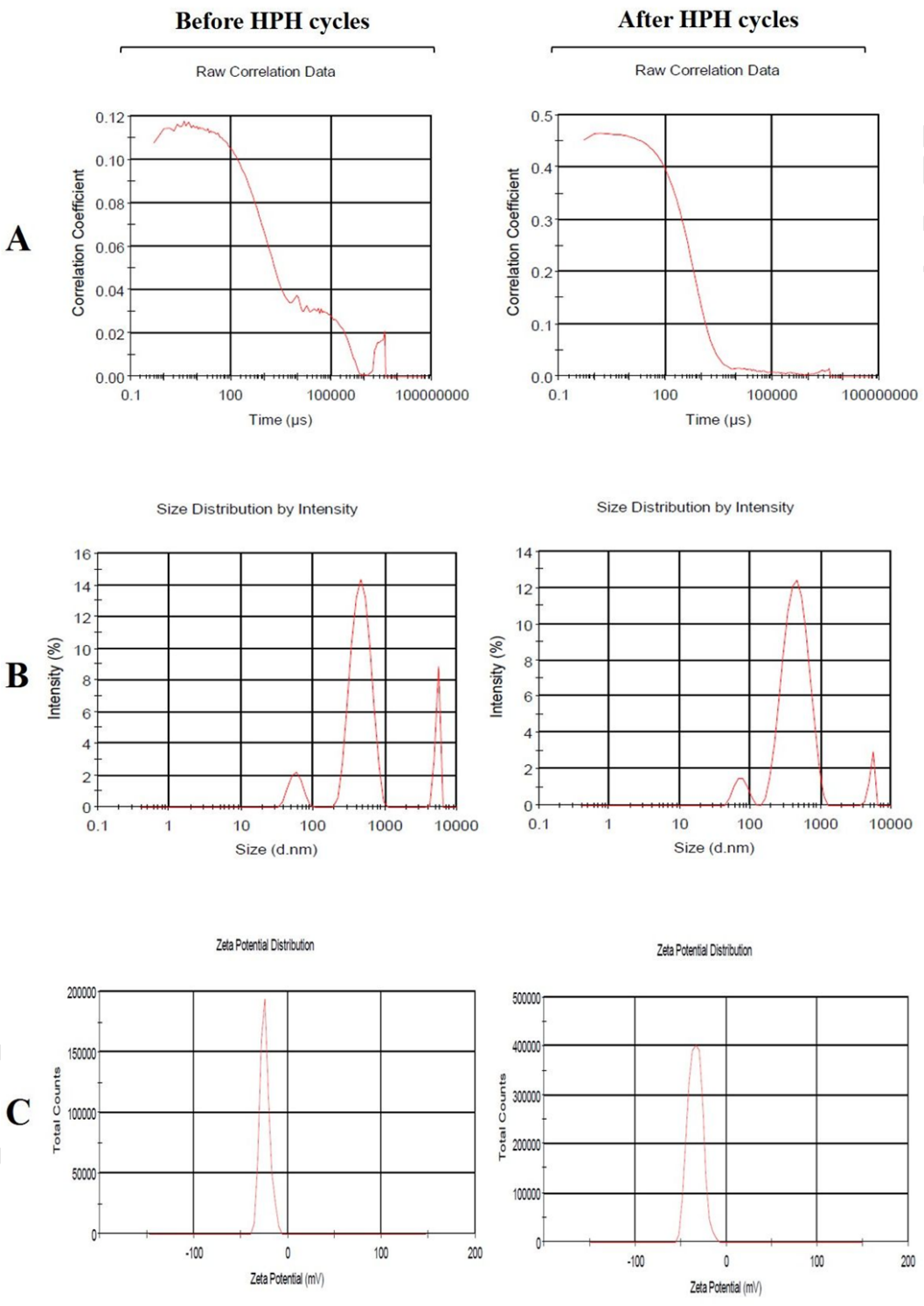
Note - ✓/×: Present/Absent Name & Signature of Investigator:

Appendix-3

H.P.H Cycles (C)	Particle size (Z-average in nm)	PDI	ZP
Before HPH	1649.33 ± 432.23	0.94 ± 0.08	-41.1 ± 3.97
5	940.37 ± 59.54	0.65 ±0.05	-35.47 ± 3.19
10	765.77 ± 30.33	0.63 ± 0.06	-33.43 ± 4.99
15	655.7 ± 67.24	0.54 ± 0.05	-33.77 ± 3.72
20	620.83 ± 24.82	0.45 ± 0.04	-32.43 ± 4.6
40	420.4 ± 11.26	0.33 ± 0.02	-30.73 ± 3.49

Particle size (Z-average), Polydispersity index (PDI), and Zeta potential (ZP) of aqueous luteolin nanosuspension (LNS) at various HPH cycles. Data expressed as mean ±S.D.

Appendix-4



(A) Raw correlation data, (B) size distribution intensity and (C) ZP distribution of LNS before HPH and after HPH cycles.

Appendix-5

APPROVAL

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	16 July 2013
Ref:	UPM/IACUC/AUP- R020/2013
	Project Title: URO PROTECTIVE FUNCTIONS OF LUTEOLIN ON CYCLOPHOSPHAMIDE INDUCED HEMORRHAGIC CYSTITIS IN RATS
Principle Investigator:	Assoc. Prof. Dr Rajesh Ramasamy
Associates:	Prof. Dr Khozirah Shaari, Dr Senthil Rajan Dharmalingam
Student:	Ravindran Harikumar
Committee decision:	The committee has reviewed and approved the proposed animal utilization protocol
AUP No:	R020/2013
Project Classification:	Chronic
Category of invasiveness:	B
Source of animals:	Laboratory Animal Management Facility, Faculty of Pharmacy, UiTM,. Puncak Alam, Malaysia
Number of Animals Approved	Fifteen (15)mice and seventy two (72) rats
Housing:	Standard rat and mouse cages
Duration:	1st August, 2013 – 31st July, 2014

(Prof. Dr. Mohd Hair Bejo)
Chairman,
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
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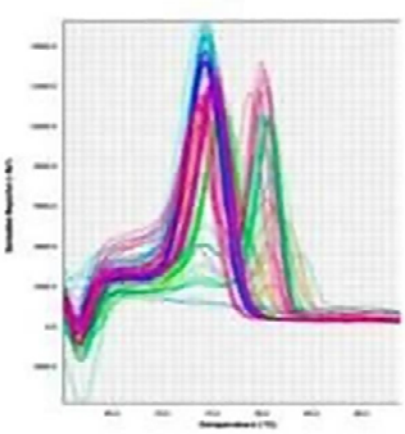
603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

Appendix-6

MELTING CURVES FOR qPCR



Experiment Information

Run Name	Two Step with Melt 2014-07-20 (1), SOD,CAT,GPX,VEGF,TNF,GAD,NFK p53,Bt
Run Start	07/20/2014 09:15:41 AM
Run Finish	07/20/2014 02:10:18 PM
Operator	FC
Notes	Harry's genes
Run On Software Version	Rotor-Gene 1.7.87
Run Signature	The Run Signature is valid.
Gain Green	8

Melt Information

Digital Filter	Light
Imported Analysis Settings	
Sample Page	Page 1
Temp. Threshold	55°c
Cycle Threshold	28

SOD CAT GPX VEG P53 BET GAD TNF

Profile

Cycle	Cycle Point
Hold @ 42°c, 30 min 0 secs	
Hold 2 @ 95°c, 10 min 0 secs	
Cycling (35 repeats)	Step 1 @ 95°c, hold 15 secs
	Step 2 @ 55°c, hold 30 secs

BIODATA OF STUDENT

Ravindran Harikumar was born in Krishnapuram, Kerala state, India, on May 10th 1974, to Mr. Ravindran Pillai and Mrs. Vijayakumari. He has a younger brother Mr. Jayakumar. He attended primary and high school in his hometown Krishnapuram. He passed pre-degree course from Mahatma Gandhi University, Kottayam, Kerala for two years (1989-1991). Following this he passed Diploma in Pharmacy (D. Pharm) conducted by Directorate of Medical Education, Government of Kerala in first class (1991-93). He successfully graduated in 1998 with degree of Bachelor of Pharmacy (B. Pharm) from Vivekananda Institute of Medical Science and Research, Salem, affiliated to Dr. M.G.R. Medical University, Tamil Nadu, India. He obtained post graduate diploma in Pharmacy practice from Annamalai University in Tamil Nadu, India and a Master degree in Pharmacognosy and Phytochemistry (M. Pharm) from College of Pharmaceutical sciences, Government Medical College Kerala with a first class. He served Blue Cross India Ltd, Kerala Health Services and currently working as a Senior Hospital Pharmacist in Government Medical College Hospital Alappuzha, Kerala (On study leave). Since 1996, his name has enrolled as registered pharmacist under Kerala Pharmacy Council. He had participated in several conferences, scientific meetings conducted by Putra Sarjana at Universiti Putra Malaysia, Kerala pharmacy council and Medical education department, Kerala.

His area of interest and passion is in the Natural Product Pharmacology. His current research includes formulation and development of phytochemicals to combat chemotherapy induced toxicities and untoward effects.

He is married to Mrs. Mayalekshmi and blessed with a girl Thanmaya (1 year).

LIST OF PUBLICATIONS

Awards and Achievements

Bronze medal - **DIAPLAST** topical formulation containing luteolin won Bronze medal at IIDEX-2015 Malaysia (Invention, Innovation & Design Expo) UiTM Puncak Alam. Nur Intan Saidaah, Siti Hajar, **Harikumar Ravindran**, Aishah Adam and Cini Mathew John.

Presented a poster - **International Immunology symposium 2015** Faculty of Medicine and Health Sciences UPM. *Luteolin Enhances Wound Healing Process in Streptozotocin (STZ) induced Diabetic Rats* - **Harikumar Ravindran**, Cini Mathew John, Bang Rom Lee, Senthil Rajan Dharmalingam, Aishah Adam and Rajesh Ramasamy. Published in *Malaysian Journal of Pathology* 2014; 36(3): 223-242.

Research publications

Ravindran Harikumar, Kesavanarayan Selvarajan, Senthilrajan Dharmalingam, Cini Mathew John, Aisha Adam, Rajesh Ramasamy. 2016. Luteolin nanosuspension ameliorates oral cyclophosphamide induced hemorrhagic cystitis in rats (Under review).

Yew Sheng Qian, **Ravindran Harikumar**, Srikanth Ventaka Meka, Senthilrajan Dharmalingam. 2015. Preparing Kaempferol Nanosuspension (KNS) using High Pressure Homogenization (HPH) technique. *BMC Proceedings*, 9 (Suppl 7).

Senthilrajan Dharmalingam, Rajkumar Madhappan, Srinivasan Ramamurthy, **Harikumar Ravindran**, S. Suresh, Senthi Kumar K. L. 2013. Antitumor Activity of *Sargassum wightii* (Greville) Extracts against Dalton's Ascites Lymphoma. *Pakistan Journal of Biological Sciences*, 16(21), 1336.

Yew S. Qian, Srinivasan Ramamurthy, Mayuren Candasamy, Shadab Md, Venkata S. Meka and **Ravindran H. Kumar** 2016. Production, Characterization and Evaluation of Kaempferol Nanosuspension for Improving Oral Bioavailability. *Current Pharmaceutical Biotechnology*, 2016, 17.

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