



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

**RAVINDRAN PILLAI HARIKUMAR**

**FPSK(p) 2016 5**



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

By

**RAVINDRAN PILLAI HARIKUMAR**

**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**

## **COPYRIGHT**

All material contained within the thesis, including without limitation text, logos, icons, photographs, and all other artwork, is copyright material of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright© Universiti Putra Malaysia



## 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

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

By

**RAVINDRAN PILLAI HARIKUMAR**

**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  $1649.3 \pm 432.23$  nm to  $420.4 \pm 11.26$  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 ( $54.41 \pm 14.49\%$ ) which was 1.5 fold higher ( $p < 0.05$ ) than pure luteolin ( $21.72 \pm 3.89\%$ ).

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- $\alpha$ , 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- $\alpha$ , 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 perlindungannya terhadap sistitis berdarah (HC) yang diaruh oleh 'cyclophosphamide' (CP) pada tikus SD.

Teknik 'high pressure homogenizer' (HPH) telah digunakan untuk menyediakan LNS. LNS yang terhasil menunjukkan kelarutan air yang tinggi serta saiz molekul yang kecil yang dikurangkan daripada  $1649.3 \pm 432.23$  nm sehingga  $420.4 \pm 11.26$  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 ( $54.41 \pm 14.49\%$ ) iaitu 2.5 kali ganda lebih tinggi ( $p < 0.05$ ) daripada luteolin tulen ( $21.72 \pm 3.89\%$ ). 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- $\alpha$ , p53, NFkB dan VEGF. Akan tetapi, rawatan CP bersama dengan LNS menunjukkan fungsi perlindungan pundi kencing dimana tahap ekspresi enzim-enzim antioksidan SOD, CAT dan GPx ditingkatkan dan ekspresi gen yang bertanggungjawab untuk inflamasi seperti TNF- $\alpha$ , 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 hepatic, 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 'reproducible' 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 fungsi anti-oksidasi, anti-inflamasi dan kimoprotektif mengurangkan 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.



## ACKNOWLEDGEMENTS

First and foremost, I wish to acknowledge my creator for governing and equipping me with the means and mechanism such that I may design and provide medicinal products which are vital and beneficial to all human kind. I acknowledge with appreciation the sort of support received from my supervisor Assoc. Prof. Dr. Rajesh Ramasamy to accept my studentship in his research team. I am so much grateful for his bolster, definite skill, noteworthy direction and budgetary support all through my entire system of study. His mentorship was central in giving a balanced steady journey to achieve profession objectives. I am not certain many graduate students are given the chance to build up their own distinction and independence by being permitted to work with such autonomy.

Much obliged to Prof. Dr. Khozirah Shaari as my co-supervisor for valuable suggestions and supports. I would like to gratefully and genuinely say thanks to Prof. Dr. Aishah Adam for her direction, comprehension, tolerance, and in particular critical comments amid my entire PhD programme at UPM. I would also like to acknowledge Dr. Dharmalingam Senthil Rajan as the co-supervisor of this study, and I am thankfully obligated to him for his exceptionally significant remarks, direction and consolation all through this programme.

Each outcome depicted in this thesis was refined with the help and backing of individual lab mates and collaborators. My earnest much gratitude goes to every one of my companions of UPM, IMU and UiTM particularly Dr. Cini, Dr. Peyman, Dr. Arunkumar, Tong, Pratheep, Shi Wei, Vahid, Satar, Maryam, Haslinda, Dr. Fazlin, Farhana, Dr. Kesav, Raghunath, Dr. Shoaib, Dr. Nisha, Velan, Ammar, Kumareswaran and others for their assistance over the span of this work. I have had a very good time in IMU research lab and thanks for Dr. Srinivasan, Dr. Srikanth and Dr. Mayuran for their in valuable help in pharmacokinetic study.

My thanks likewise go to the whole lab mates of Immunology lab, UPM, Research lab IMU, Bukit Jalil and UiTM, Puncak Alam for their various assistances. I wish to extend my heartfelt appreciation to the numerous individuals who served to convey this research to fulfilment.

At long last, I must express my exceedingly momentous admiration to my parents, brothers, to my wife Mayalekshmi and our little blessed angel Thanmaya for providing me with unfailing backing and nonstop consolation during my time of study and through the way of inquiring about and writing this thesis. Her encouragement, consolation, calm tolerance and steady love were obviously the bedrock whereupon the previous years of my life have been created. This achievement would not have been conceivable without them. Much obliged to you. I wish to extent my gratitude to all my family members and friends, thank you all.

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)

**Khozirah Shaari, PhD**

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**

Senior Lecturer  
Faculty of Pharmacy  
International Medical University  
(Member)

---

**BUJANG BIN KIM HUAT, PhD**

Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:

## Declaration by Members of Supervisory Committee

This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: \_\_\_\_\_  
Name of  
Chairman of  
Supervisory  
Committee: Assoc.Prof. Dr. Rajesh Ramasamy,

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

## TABLE OF CONTENTS

	Page
<b>ABSTRACT</b>	i
<b>ABSTRAK</b>	iii
<b>ACKNOWLEDGEMENTS</b>	v
<b>APPROVAL</b>	vi
<b>DECLARATION</b>	viii
<b>LIST OF TABLES</b>	xiv
<b>LIST OF FIGURES</b>	xv
<b>LIST OF ABBREVIATIONS</b>	xvi
 <b>CHAPTER</b>	
 <b>1 INTRODUCTION</b>	 1
1.1 Problem statement	3
1.2 Research hypothesis	4
1.3 General objective	4
1.4 Specific objective	4
 <b>2 LITERATURE REVIEW</b>	 5
2.1 Flavonoids	5
2.1.1 Pharmacological properties of flavonoids	6
2.1.2 Flavonoids and its bioavailability enhancement	7
2.2 Luteolin	9
2.2.1 Luteolin-distribution and pharmacological properties	10
2.2.2 Luteolin's oral bioavailability and metabolism	13
2.3 Enhancement of bioavailability of orally administered drugs	14
2.3.1 Need for bioavailability enhancement	14
2.3.2 Drug delivery systems to improve bioavailability	14
2.3.3 Oral drug formulation methods based on nanotechnology	15
2.3.4 Nanosuspensions for oral administration	16
2.3.4.1 HPH technique	16
2.3.4.2 Dissocubes technology	17
2.3.5 Characterization of nanosuspensions	18
2.3.5.1 Analysis of particle size and size distribution	18
2.3.5.2 Morphology of nanosuspensions	19
2.3.5.3 Evaluation of sedimentation and redispersion	19
2.3.5.4 Particle surface charge	19
2.3.5.5 Analysis of crystal structure	20
2.3.5.6 Assessment of chemical properties	20
2.4 Reverse phase chromatography/ RP-HPLC	20
2.5 Pharmacokinetics of formulations (PK)	21
2.5.1 Definition	21

2.5.2	Clinical pharmacokinetics	22
2.5.3	Drug concentration in biological samples	22
2.5.4	Drug plasma concentration - time curve	23
2.5.5	Types of pharmacokinetics study models	23
2.5.6	Pharmacokinetic parameters	24
2.6	Cyclophosphamide - pharmacology and toxicology	25
2.6.1	Cyclophosphamide mediated Haemorrhagic cystitis	26
2.6.2	Mechanism of CP induced urotoxicity	27
2.6.3	Pathogenesis of HC	30
2.6.4	Function of transcriptional factors and others in HC	31
2.6.4.1	Nuclear Factor $\kappa$ B	32
2.6.4.2	Tumor necrosis factor $\alpha$	32
2.6.4.3	Tumor suppressor protein	33
2.6.4.4	Growth arrest and DNA damage inducible protein- $\alpha$	34
2.7	Natural products and prevention of CP induced urotoxicity	36
<b>3.</b>	<b>FORMULATION AND CHARACTERIZATION OF LUTEOLIN NANOSUSPENSION (LNS)</b>	<b>36</b>
3.1	Introduction	36
3.2	Materials and Methods	38
3.2.1	Drugs and chemicals	38
3.2.2	Formulation of LNS	38
3.2.3	Lyophilization	38
3.2.4	Re-dispersion and phase separation of LNS	38
3.2.5	Characterization of nanosuspension	39
3.2.6	Morphological evaluation of LNS by TEM	39
3.2.7	Fourier transform infrared spectroscopy	39
3.2.8	X-ray Diffractometry (XRD)	40
3.2.9	Nuclear magnetic resonance spectroscopy (NMR)	40
3.2.10	Acute toxicity study	40
3.2.11	Statistical analysis	41
3.3	Results	41
3.3.1	Particle size, morphology and Zeta potential of LNS	41
3.3.2	Morphological evaluation of LNS by TEM	42
3.3.3	Lyophilization and re-dispersability	44
3.3.4	Short term stability study	44
3.3.5	FTIR analysis of LNS	45
3.3.6	X-ray powder diffraction measurements	46
3.3.7	Nuclear magnetic resonance spectroscopy (NMR)	48
3.3.8	Acute toxicity	49
3.3.8.1	Survival and clinical sign	49
3.3.8.2	Change in bodyweight gain	49
3.3.8.3	Histology	52
3.4	Discussion	52
3.5	Conclusion	55

<b>4.</b>	<b>IN VIVO PHARMACOKINETICS AND RELATIVE BIOAVAILABILITY STUDY OF LNS</b>	<b>56</b>
4.1	Introduction	56
4.2	Materials and Methods	57
4.2.1	Chemicals	57
4.2.2	Instrumentation	57
4.2.3	Animals	57
4.2.4	Chromatographic conditions	57
4.2.5	Method validation for pharmacokinetics study	58
4.2.6	Relative bioavailability and pharmacokinetics of LNS	58
4.2.7	Non-compartmental pharmacokinetic analysis	59
4.2.8	Data analysis and statistics	59
4.3	RESULTS	60
4.3.1	HPLC condition and internal standard selection	60
4.3.2	Pharmacokinetic profile of LNS	61
4.4	Discussion	64
4.5	Conclusion	66
<b>5.</b>	<b>URO PROTECTIVE FUNCTION OF LUTEOLIN NANOSUSPENSION</b>	<b>67</b>
5.1	Introduction	67
5.2	Materials and Methods	68
5.2.1	Drugs and chemicals	68
5.2.2	Animals	68
5.2.3	Experimental design	69
5.2.4	Urine analysis	69
5.2.5	Measurement of body weight and relative organ weights	70
5.2.6	Morphological evaluation	70
5.2.7	Biochemical analysis	71
5.2.8	Serum cytokines and antioxidant enzymes analysis	71
5.2.9	Histopathological analysis	71
5.2.10	Statistical analysis	72
5.3	Results	72
5.3.1	Confirmation of CP induced HC by urine analysis	72
5.3.2	Effect of LNS on reduction of body weight in HC	73
5.3.3	Supplementation of LNS preserves relative organ weight	74
5.3.4	LNS improves morphology of urinary bladder in HC	76
5.3.5	LNS normalizes the urine composition in HC	76
5.3.6	LNS promotes reconstitution of cytokines and enzymes	78
5.3.7	Estimation of hepatic toxicity marker enzyme levels	81
5.3.8	LNS prevents CP induced bladder tissue damages	84
5.4	Discussion	86

5.5	Conclusion	89
<b>6.</b>	<b>ROLE OF INFLAMMATION REGULATING GENES IN MEDIATING PROTECTIVE EFFECT OF LNS IN HC</b>	<b>90</b>
6.1	Introduction	90
6.2	Materials and Methods	92
6.2.1	Experimental design	92
6.2.2	Measurement of gene expression by at urinary bladder	92
6.2.3	Statistical analysis	93
6.3	Results	95
6.3.1	LNS upregulates SOD genes in urinary bladder	95
6.3.2	LNS upregulates CAT genes in urinary bladder	95
6.3.3	LNS upregulates GPx genes in urinary bladder	96
6.3.4	LNS upregulates GADD45 $\alpha$ genes in urinary bladder	97
6.3.5	LNS down regulates <i>p53</i> genes in urinary bladder	98
6.3.6	LNS treatment inhibits TNF- $\alpha$ in urinary bladder	99
6.3.7	LNS treatment inhibits NF $\kappa$ B in urinary bladder	100
6.3.8	LNS treatment inhibits production of VEGF	101
6.4	Discussion	102
6.5	Conclusion	105
<b>7</b>	<b>SUMMARY, CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH</b>	<b>106</b>
7.1	Summary	106
7.2	Conclusion	107
7.3	Limitations and Further Recommendations	108
	<b>REFERENCES</b>	<b>109</b>
	<b>APPENDICES</b>	<b>135</b>
	<b>BIODATA OF STUDENT</b>	<b>141</b>
	<b>LIST OF PUBLICATIONS</b>	<b>142</b>

## LIST OF TABLES

Table		Page
4.1	Plasma concentration of LNS	63
4.2	Plasma concentration of luteolin	63
4.3	Relative bioavailability and pharmacokinetics values	64
5.1	Treatment given to different urinary group	69
5.2	Grading of bladder lesions	70
5.3	Urine analysis report	72
5.4	Morphology analysis of urinary bladder	76
6.1	Gene primer sequences	94



## LIST OF FIGURES

Figure		Page
2.1	Flavonoids- structure and numbering pattern	6
2.2	Luteolin and apigenin with glycosides	7
2.3	Structure of luteolin	9
2.4	Luteolin cleaves ROS	10
2.5	Plasma-time curve	23
2.6	Structure of cyclophosphamide and its metabolites	26
2.7	Metabolism of CP	29
2.8	Chemical structure of MESNA	30
3.1	HPH technique	37
3.2	Morphology and microscopy of LNS and luteolin	42
3.3	Particle size, PDI and ZP of luteolin	43
3.4	PDI, ZP and particle size during freeze drying	44
3.5	Storage stability of LNS	45
3.6	FTIR analysis	46
3.7	X-ray diffractogram	47
3.8	NMR spectroscopy analysis	48
3.9	Change in body weight gain-acute toxicity	49
3.10	Histology of kidney, liver and pancreas	50
3.11	Histology of spleen, urinary bladder and thymus	51
4.1	Luteolin and kaempferol structure	60
4.2	Chromatogram of samples	61
4.3	Luteolin standard curve	62
4.4	Concentration-time curve of luteolin and LNS	62
5.1	Change in body weight gain	73
5.2A	Relative weight of kidney	74
5.2B	Relative weight of liver	75
5.2.C	Relative weight of urinary bladder	75
5.3	Morphology of urinary bladder	77
5.4	Estimation of albumin, creatinine, urea and total protein	78
5.5	Serum levels of Interleukins	79
5.6A	Serum SOD activity	81

5.6B	Serum CAT activity	81
5.6C	Serum GPx activity	81
5.7	Serum enzyme levels	83
5.8	Histology of urinary bladder	85
6.1	mRNA level of SOD gene	95
6.2	mRNA level of CAT gene	96
6.3	mRNA level of GPx gene	97
6.4	mRNA level of GADD45A gene	98
6.5	mRNA level of <i>p53</i> gene	99
6.6	mRNA level of TNF- $\alpha$ gene	100
6.7	mRNA level of NF $\kappa$ B gene	101
6.8	mRNA level of VEGF gene	102
6.9	Schematic representation of uroprotective activity of LNS	105

## LIST OF ABBREVIATIONS

AUC	Area under curve
AUMC	Area under moment curve
CAT	Catalase
C <sub>max</sub>	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 <sub>A</sub>	Alternative hypothesis
H <sub>o</sub>	Null hypothesis
HPH	High pressure homogenization
ICAM	Intercellular adhesion molecule
K <sub>el</sub>	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	Phosphoramidate 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- $\alpha$	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.



## REFERENCES

- Adams, J.D and Klaidman, L.K. (1993). Acrolein-induced oxygen radical formation. *Free radical Biology and Medicine*, 15(2), 187-193.
- Aghi, M., Hochberg., Breakefield, X.O. (2000). Prodrug activation enzymes in cancer gene therapy. *The Journal of Gene Medicine*, 2(3), 148-164.
- Aguilar, M.I and Hearn, M. (1996). High-resolution reversed-phase high-performance liquid chromatography of peptides and proteins. *Methods in Enzymology*, 3(1), 270-278.
- Ain, A. and Gupta, P.K. (2008). Effect of arginine hydrochloride and hydroxypropyl cellulose as stabilizers on the physical stability of high drug loading nanosuspensions of a poorly soluble compound. *International Journal of Pharmaceutics*, 351(1), 282-288.
- Alarcon, R. and Meienhofer, J. (1971). Formation of the cytotoxic aldehyde acrolein during in vitro degradation of cyclophosphamide. *Nature*, 233(42), 250-252.
- Albanese, A., Tang., Chan, W.C. (2012). The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annual Review of Biomedical Engineering*, 14, 1-16.
- Allan, A.C., Hellens, R.P., Laing, W.A. (2008). MYB transcription factors that colour our fruit. *Trends in Plant Science*, 13(3), 99-102.
- Amidon, G., Lennernäs, L., Shah, V.P., Crison, J.R. (1995). A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharmaceutical Research*, 12(3), 413-420.
- Anderson, D., Bishop, J.B., Garner, R., Ostrosky-Wegman, P., Selby, P.B. (1995). Cyclophosphamide: review of its mutagenicity for an assessment of potential germ cell risks. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 330(1), 115-181.
- Anderson, R., Amarasinghe, Fisher, L., Mak, W.B., Packer, J.E. (2000). Reduction in free-radical-induced DNA strand breaks and base damage through fast chemical repair by flavonoids. *Free Radical Research*, 33(1), 91-103.
- Ando, C., Takahashi, Hirai, S., Nishimura, Lin, S., Uemura, T.... (2009). Luteolin, a food-derived flavonoid, suppresses adipocyte-dependent activation of macrophages by inhibiting JNK activation. *FEBS Letters*, 583(22), 3649-3654.

- Angulo, I., Jimenez-Diaz, M.B., Garcia-Bustos, J.F., Gargallo, F.G., Munoz-Fernandez, M.A.... (2002). *Candida albicans* infection enhances immunosuppression induced by cyclophosphamide by selective priming of suppressive myeloid progenitors for NO production. *Cellular Immunology*, 218(1-2), 46-58.
- Arnold, H., Bourseaux, F., Brock, N. (1958). Novel cancer chemotherapeutic agents from the group of cyclic nitrogen mustard Phosphamidester. *Natural sciences*, 45(3), 64-66.
- Arts, I.C., Hollman, P.C., Feskens, E.J., Mesquita, H.B.B., Kromhout, D. (2001). Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *The American Journal of Clinical Nutrition*, 74(2), 227-232.
- Aungst, B.J. (1993). Novel formulation strategies for improving oral bioavailability of drugs with poor membrane permeation or presystemic metabolism. *Journal of Pharmaceutical Sciences*, 82(10), 979-987.
- Balamurugan, K. and Karthikeyan, J. (2012). Evaluation of the antioxidant and anti-inflammatory nature of luteolin in experimentally induced hepatocellular carcinoma. *Biomedicine and Preventive Nutrition*, 2(2), 86-90.
- Bansal, S., Bansal, M., Kumria, R. (2012). Nanocrystals: current strategies and trends. *International Journal of Research in Pharmaceutical and Biomedical Sciences*, 3(1), 406-419.
- Bansal, S.S., Goel, M., Aqil, F., Vadhanam, M.V. and Gupta, R.C. (2011). Advanced drug delivery systems of curcumin for cancer chemoprevention. *Cancer Prevention Research*, 4(8), 1158-1171.
- Beg, S.S., Rizwan, S., Irfanuddin, M., Shobha, M.D. (2011). Bioavailability enhancement strategies: basics, formulation approaches and regulatory considerations. *Current Drug Delivery*, 8(6), 691-702.
- Beyer, B.M., de Voogt, H. and Schaberg, A. (1978). The effects of cyclophosphamide treatment on the epithelium and stroma of the urinary bladder. *European Journal of Cancer* (1965), 14(10), 1029-1035.
- Bhatia, K., Ahmad, F., Rashid, H., Raisuddin, S. (2008). Protective effect of S-allylcysteine against cyclophosphamide-induced bladder hemorrhagic cystitis in mice. *Food and Chemical Toxicology*, 46(11), 3368-3374.
- Bhatia, K., Kaur, M., Atif, F., Ali, M., Rehman, H., Rahman, S.... (2006). Aqueous extract of *Trigonella foenum-graecum* L. ameliorates additive

urotoxicity of buthionine sulfoximine and cyclophosphamide in mice. *Food and Chemical Toxicology*, 44(10), 1744-1750.

BioVision - Life Science Source | 800-891-9699. (n.d.). Retrieved July 11, 2014, from <http://www.biovision.com>.

Block, G., Patterson, B., Subar, A. (1992). Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutrition and Cancer*, 18(1), 1-29.

Boeira, V.T., Leite, C.E., Santos Jr, A.A., Edelweiss, M.I., Calixto, J.B., Campos, M.M.... (2011). Effects of the hydroalcoholic extract of *Phyllanthus niruri* and its isolated compounds on cyclophosphamide-induced hemorrhagic cystitis in mouse. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 384(3), 265-275.

Boyd, V.L., Robbins, J.D., Egan, W., Ludeman, S.M. (1986). Phosphorus-31 nuclear magnetic resonance spectroscopic observation of the intracellular transformations of oncostatic cyclophosphamide metabolites. *Journal of Medicinal Chemistry*, 29(7), 1206-1210.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews*, 56(11), 317-333.

Brock, N. and Pohl, J. (1983). The development of mesna for regional detoxification. *Cancer Treatment Reviews*, 10, 33-43.

Brock, N., Pohl, J., Stekar, J. (1981). Detoxification of urotoxic oxazaphosphorines by sulfhydryl compounds. *Journal of Cancer Research and Clinical Oncology*, 100(3), 311-320.

Brown, J.E., Khodr, H., Hider, R.C., Rice-Evans, C.A. (1998). Structural dependence of flavonoid interactions with Cu<sup>2+</sup> ions: implications for their antioxidant properties. *Biochemistry Journal*, 330(Pt 3), 1173-1178.

Brusick, D. (1993). Genotoxicity of phenolic antioxidants. *Toxicology and Industrial Health*, 9(1-2), 223.

Bulitta, J.B. and Holford, N.H. (2008). Non-Compartmental Analysis. *Wiley Encyclopedia of Clinical Trials*. Published Online: 13 June 2008.

Burr, M.L. (1995). Explaining the French paradox. *Journal Royal Society Health*, 115(4), 217-219.

Cai, Q., Rahn, R.O., Zhang, R. (1997). Dietary flavonoids, quercetin, luteolin and genistein, reduce oxidative DNA damage and lipid peroxidation and quench free radicals. *Cancer Letters*, 119(1), 99-107.

- Campobasso, O. and Berrino, F. (1972). Early effects of cyclophosphamide on mouse bladder epithelium. *Pathobiology*, 38(2), 144-157.
- Cao, G., Sofic, E. and Prior, R.L. (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biology and Medicine*, 22(5), 749-760.
- Cárdenas, M., Marder, M., Blank, V.C. and Roguin, L.P. (2006). Antitumor activity of some natural flavonoids and synthetic derivatives on various human and murine cancer cell lines. *Bioorganic and Medicinal Chemistry*, 14(9), 2966-2971.
- Chan, H.K. and Kwok, P.C.L. (2011). Production methods for nanodrug particles using the bottom-up approach. *Advanced Drug Delivery Reviews*, 63(6), 406-416.
- ChenBi, A., Dong, X., Jiang, Y., Rui, B., Liu, J.... (2014). Luteolin exhibits anti-inflammatory effects by blocking the activity of heat shock protein 90 in macrophages. *Biochemical and Biophysical Research Communications*, 443(1), 326-332.
- Chen, K., Song, F., Li, L., Jiang, H. (2012). Pharmacokinetic study of luteolin, apigenin, chrysoeriol and diosmetin after oral administration of Flos Chrysanthemi extract in rats. *Fitoterapia*, 83(8), 1616-1622.
- Chen,T., Li, L.P., Lu, X.Y, Jiang, H.D., Zeng, S. (2007). Absorption and excretion of luteolin and apigenin in rats after oral administration of *Chrysanthemum morifolium* extract. *Journal of Agricultural and Food Chemistry*, 55(2), 273-277.
- Chen Peng, W.H., Tsai, K.D., Hsu, S.L. (2007). Luteolin suppresses inflammation-associated gene expression by blocking NF- $\kappa$ B and AP-1 activation pathway in mouse alveolar macrophages. *Life Sciences*, 81(23), 1602-1614.
- Chen, T., Sun, S., Kong, S., Wang, Y., Ye, J.... (2012). The exposure of luteolin is much lower than that of apigenin in oral administration of Flos Chrysanthemi extract to rats. *Drug Metabolism and Pharmacokinetics*, 27(1), 162-168.
- Chen, C.S., Lin, J.T., Goss, K.A., He, Y., Halpert, J.R., Waxman, D.J. (2004). Activation of the anticancer prodrugs cyclophosphamide and ifosfamide: identification of cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics. *Molecular Pharmacology*, 65(5), 1278-1285.
- Chingunpituk, J. (2011). Nanosuspension technology for drug delivery. *Walailak Journal of Science and Technology*, 4(2), 139-153.

- Chung, F.L., Schwartz, J., Herzog, C.R., Yang, Y.M. (2003). Tea and cancer prevention: studies in animals and humans. *Journal of Nutrition*, 133(10), 3268-3274.
- Coggins, P.R., Ravdin, R.G., Eisman, S. H. (1960). Clinical evaluation of a new alkylating agent: cytoxan (cyclophosphamide). *Cancer*, 13, 1254-1260.
- Cohen, J.L. and Jao, J.Y. (1970). Enzymatic basis of cyclophosphamide activation by hepatic microsomes of the rat. *Journal Pharmacology Experimental Therapeutics*, 174(2), 206-210.
- Colvin, O. (1999). An overview of cyclophosphamide development and clinical applications. *Current Pharmaceutical Design*, 5, 555-560.
- Conner, E.M. and Grisham, M.B. (1996). Inflammation, free radicals, and antioxidants. *Nutrition*, 12(4), 274-277.
- Cook, N. and Samman, S. (1996). Flavonoids-chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry*, 7(2), 66-76.
- Covaliu, C.I., Matei, C.I., Anculescu, A., Jitaru, I. and Berger, D. (2009). Fe<sub>3</sub>O<sub>4</sub> and CoFe<sub>2</sub>O<sub>4</sub> nanoparticles stabilized in sodium alginate polymer. *UPB Scientific Bulletin. B*, 71(4), 53-60.
- Cox, P.J. (1979). Cyclophosphamide cystitis-identification of acrolein as the causative agent. *Biochemical Pharmacology*, 28(13), 2045-2049.
- Crocitto, L., Simpson, J., Wilson, T. (1996). Bladder augmentation in the prevention of cyclophosphamide-induced haemorrhagic cystitis in the rat model. *British Journal of Urology*, 78(4), 530-533.
- Cuesta, S., Kireev, R., Forman, K., Escames, G., Ariznavarreta, C., Vara, E.... (2010). Melatonin improves inflammation processes in liver of senescence-accelerated prone male mice (SAMP8). *Experimental Gerontology*, 45(12), 950-956.
- Dai, L., Cheng, H., Li, W. (1985). The influence<sup>TM</sup> 70 of luteolin on experimental inflammatory models in rats. *Acta Anhui Medical University* 1985, 20, 1-3.
- Dang, H., Meng, M., Zhao, H., Iqbal, J., Dai, R., Deng, Y.... (2014). Luteolin-loaded solid lipid nanoparticles synthesis, characterization and improvement of bioavailability, pharmacokinetics in vitro and vivo studies. *Journal of Nanoparticle Research*, 16(4), 1-10.
- Dantas, A.C., Batista-Júnior, F.A., Macedo, L.F., Mendes, M.N.C., Azevedo, Í.M., Medeiros, A.C. (2010). Protective effect of simvastatin in the



cyclophosphamide-induced hemorrhagic cystitis in rats. *Acta Cirurgica Brasileira*, 25(1), 43-46.

Das, U.B., Mallick, M., Debnath, J.M., Ghosh, D. (2002). Protective effect of ascorbic acid on cyclophosphamide-induced testicular gametogenic and androgenic disorders in male rats. *Asian Journal of Andrology*, 4(3), 201-208.

Dasika, G.K., Lin, S.C.J., Zhao, S., Sung, P., Tomkinson, A., Lee, E.Y.P. (1999). DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. *Oncogene*, 18(55), 7883-7899.

De Villiers, M.M., Aramwit, P., Kwon, G.S. (2008). Nanotechnology in drug delivery: Springer Science & Business Media. 22(12), 12-18.

Derendorf, H., Möllmann, H., Barth, J., Möllmann, C., Tunn, S., Krieg, M. (1991). Pharmacokinetics and oral bioavailability of hydrocortisone. *The Journal of Clinical Pharmacology*, 31(5), 473-476.

DeVries, C. and Freiha, F. (1990). Hemorrhagic cystitis: a review. *The Journal of urology*, 143(1), 1-9.

Dobrek, L. and Thor, P.J. (2012). Bladder urotoxicity pathophysiology induced by the oxazaphosphorine alkylating agents and its chemoprevention. *Postepy Hig Med Dosw (Online)*, 66, 592-602.

Doherty, A.T., Hayes, J.E., Molloy, J., Wood, C., O'Donovan, M.R. (2013). Bone marrow micronucleus frequencies in the rat after oral administration of cyclophosphamide, hexamethylphosphoramide or gemifloxacin for 2 and 28 days. *Toxicology Research*, 2(5), 321-327.

Domitrović, R., Cvijanović, O., Pugel, E.P., Zagorac, G.B., Mahmutefendić, H., Škoda, M. (2013). Luteolin ameliorates cisplatin-induced nephrotoxicity in mice through inhibition of platinum accumulation, inflammation and apoptosis in the kidney. *Toxicology*, 310(0), 115-123.

Duthie, G. and Crozier, A. (2000). Plant-derived phenolic antioxidants. *Current Opinion in Lipidology*, 11(1), 43-47.

Ehrhardt, R.O., Lúdvíksson, B.R., Gray, B., Neurath, M., Strober, W. (1997). Induction and prevention of colonic inflammation in IL-2-deficient mice. *The Journal of Immunology*, 158(2), 566-573.

Essner, R., Rhoades, K., McBride, W.H., Morton, D.L., Economou, J. (1989). IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. *The Journal of Immunology*, 142(11), 3857-3861.

- Eumkeb, G., Siriwong, S., Thumanu, K. (2012). Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action. *Journal Photochemistry Photobiology B*, 117, 247-253.
- Exley, A., Cohen, J., Buurman, W., Owen, R., Lumley, J., Hanson, G.... (1990). Monoclonal antibody to TNF in severe septic shock. *The Lancet*, 335(8700), 1275-1277.
- Fasinu, P., Pillay, V., Ndesendo, V.M., du Toit, L.C., Choonara, Y.E. (2011). Diverse approaches for the enhancement of oral drug bioavailability. *Biopharmaceutics and Drug Disposition*, 32(4), 185-209.
- Fiander, H. and Schneider, H. (2000). Dietary ortho phenols that induce glutathione S-transferase and increase the resistance of cells to hydrogen peroxide are potential cancer chemopreventives that act by two mechanisms: the alleviation of oxidative stress and the detoxification of mutagenic xenobiotics. *Cancer Letters*, 156(2), 117-124.
- Finkel, T. (2005). Radical medicine: treating ageing to cure disease. *Nature Reviews Molecular Cell Biology*, 6(12), 971-976.
- Fisher, D.E. (1994). Apoptosis in cancer therapy: crossing the threshold. *Cell*, 78(4), 539-542.
- Fleming, R.A. (1997). An overview of cyclophosphamide and ifosfamide pharmacology. *Pharmacotherapy*, 17(5), 146-154.
- Fornace, A. J., Alamo, I., Hollander, M.C. and Lamoreaux, E. (1989). Induction of heat shock protein transcripts and B2 transcripts by various stresses in Chinese hamster cells. *Experimental Cell Research*, 182(1), 61-74.
- Fraiser, Kanekal, S. and Kehrer, J.P. (1991). Cyclophosphamide toxicity. *Drugs*, 42(5), 781-795.
- Funakoshi, T., Nakamura, K., Tago, K., Mashino, T., Kasahara, T. (2011). Anti-inflammatory activity of structurally related flavonoids, Apigenin, Luteolin and Fisetin. *International Immunopharmacology*, 11(9), 1150-1159.
- Gabrielsson, J. and Weiner, D. (2012). Non-compartmental analysis. *Computational Toxicology*: 1(1), 377-389.
- Ganta, S., Paxton, J.W., Baguley, B.C., Garg, S. (2009). Formulation and pharmacokinetic evaluation of an asulacrine nanocrystalline suspension for intravenous delivery. *International Journal of Pharmaceutics*, 367(1), 179-186.

- Ghosh, D., Das, U., Ghosh, S., Mallick, M., Debnath, J. (2002). Testicular gametogenic and steroidogenic activities in cyclophosphamide treated rat: a correlative study with testicular oxidative stress. *Drug and Chemical Toxicology*, 25(3), 281-292.
- Giliyar, C., Fikstad, D., Tyavanagimatt, S. (2006). Challenges and opportunities in oral delivery of poorly water-soluble drugs. *Drug Delivery Technology*, 1(6), 57-63.
- Gillette, J.R. (1971). Factors affecting drug metabolism. *Annals of the New York Academy of Sciences*, 179(1), 43-66.
- Golden, E., Pellicciotta, I., Demaria, S., Barcellos-Hoff, M.H., Formenti, S.C. (2012). The convergence of radiation and immunogenic cell death signaling pathways. *Frontiers in Oncology*, 2, 88.
- Gomes, T., Santos, C., Souza-Filho, M., Cunha, F., Ribeiro, R. (1995). Participation of TNF-alpha and IL-1 in the pathogenesis of cyclophosphamide-induced hemorrhagic cystitis. *Brazilian Journal of Medical and Biological Research*, 28(10), 1103-1108.
- Gorczyńska, E., Turkiewicz, D., Rybka, K., Toporski, J., Kalwak, K., Dyla, A.... (2005). Incidence, clinical outcome, and management of virus-induced hemorrhagic cystitis in children and adolescents after allogeneic hematopoietic cell transplantation. *Biology of Blood and Marrow Transplantation*, 11(10), 797-804.
- Gray, K.J., Engelmann, U.H., Johnson, E., Fishman, I. (1986). Evaluation of misoprostol cytoprotection of the bladder with cyclophosphamide (Cytoxan) therapy. *The Journal of Urology*, 136(2), 497-500.
- Griffiths, L.A. (1982). Mammalian metabolism of flavonoids *The flavonoids* (pp. 681-718): Springer.
- Guide for the Care and Use of Laboratory Animals. (1996). The National Academies Press.
- Guideline, OECD. (2001). 423: Acute oral toxicity-acute toxic class method. *OECD Guidelines for the Testing of Chemicals*. OECD. OECD Publishing, Paris, France, 1-14.
- Gunasekaran, T., Haile, T., Nigusse, T., Dhanaraju, M.D. (2014). Nanotechnology: an effective tool for enhancing bioavailability and bioactivity of phytomedicine. *Asian Pacific Journal of Tropical Biomedicine*, 4, S1-S7.
- Hahn, N.M. (2009). Learning to control cyclophosphamide induced cystitis. *The Journal of Urology*, 181(5), 1987-1988.



- Haldar, S., Dru, C., Bhowmick, N.A. (2014). Mechanisms of hemorrhagic cystitis. *American Journal of Clinical and Experimental Urology*, 2(3), 199.
- Halliwell, B. (1994). Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *The Lancet*, 344(8924), 721-724.
- Hamsa, T. and Kuttan, G. (2012). Tinospora cordifolia ameliorates urotoxic effect of cyclophosphamide by modulating GSH and cytokine levels. *Experimental and Toxicologic Pathology*, 64(4), 307-314.
- Han, M., Liu, X., Guo, Y., Wang, Y., Wang, X. (2013). Preparation, characterization, biodistribution and antitumor efficacy of hydroxycamptothecin nanosuspensions. *International Journal of Pharmaceutics*, 455(1), 85-92.
- Harris, C.C. (1996). Structure and function of the *p53* tumor suppressor gene: clues for rational cancer therapeutic strategies. *Journal of the National Cancer Institute*, 88(20), 1442-1455.
- Havsteen. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology and Therapeutics*, 96(2), 67-202.
- Heim, K.E., Tagliaferro, A.R., Bobilya, D.J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry*, 13(10), 572-584.
- Hirano, T., Arimitsu, J., Higa, S., Naka, T., Ogata, A., Shima, Y.... (2006). Luteolin, a flavonoid, inhibits CD40 ligand expression by activated human basophils. *International Archives of Allergy Immunology*, 140(2), 150-156.
- Hirose, T., Sowa, Y., Takahashi, S., Saito, S., Yasuda, C., Shindo, N.... (2003). *p53*-independent induction of Gadd45 by histone deacetylase inhibitor: coordinate regulation by transcription factors Oct-1 and NF- $\kappa$ B. *Oncogene*, 22(49), 7762-7773.
- Hollander, M.C., Zhan, Q., Bae, I., Fornace, A.J. (1997). Mammalian GADD34, an apoptosis-and DNA damage-inducible gene. *Journal of Biological Chemistry*, 272(21), 13731-13737.
- Hollman, P.C. (2004). Absorption, bioavailability, and metabolism of flavonoids. *Pharmaceutical Biology*, 42(suppl 1), 74-83.
- Hollman, P.C., Bijlsman, M.N., van Gameren, Y., Cnossen, E.P., de Vries, J.H., Katan, M.B. (1999). The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research*, 31(6), 569-573.

- Hollman, P.C.H. and Katan, M. (1999). Dietary flavonoids: intake, health effects and bioavailability. *Food and Chemical Toxicology*, 37(9), 937-942.
- Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R.... (1996). Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucleic Acids Research*, 24(1), 141-146.
- Hommes, D., Peppelenbosch, M., van Deventer, S. (2003). Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. *Gut*, 52(1), 144-151.
- Horvathova, K., Novotný, L., Tothova, D., Vachalkova, A. (2003). Determination of free radical scavenging activity of quercetin, rutin, luteolin and apigenin in H<sub>2</sub>O<sub>2</sub>-treated human ML cells K562. *Neoplasma*, 51(5), 395-399.
- Hotter, G., Closa, D., Prats, N., Pi, F., Gelpí, E., Roselló-Catafau, J. (1997). Free radical enhancement promotes leucocyte recruitment through a PAF and LTB<sub>4</sub> dependent mechanism. *Free Radical Biology and Medicine*, 22(6), 947-954.
- Hu, C. and Kitts, D.D. (2004). Luteolin and luteolin-7-O-glucoside from dandelion flower suppress iNOS and COX-2 in RAW264.7 cells. *Molecular and Cellular Biochemistry*, 265(1-2), 107-113.
- Ilbey, Y.O., Ozbek, E., Simsek, A., Otunctemur, A., Cekmen, M., Somay, A. (2009). Potential chemoprotective effect of melatonin in cyclophosphamide-and cisplatin-induced testicular damage in rats. *Fertility and Sterility*, 92(3), 1124-1132.
- Jaijoy, K., Soonthornchareonnon, N., Lertprasertsuke, N., Panthong, A., Sireeratawong, S. (2010). Acute and chronic oral toxicity of standardized water extract from the fruit of *Phyllanthus emblica* Linn. *International Journal of Applied Research in Natural Products*, 3(1), 48-58.
- Jambhekar, S.S. and Breen, P.J. (2009). Basic pharmacokinetics: Pharmaceutical press.
- Jandera, P. (2011). Stationary and mobile phases in hydrophilic interaction chromatography: a review. *Analytica Chimica Acta*, 692(1), 1-25.
- Jeon, I.H., Kim, H.S., Kang, H.J., Lee, H.S., Jeong, S.I., Kim, S.J.... (2014). Anti-inflammatory and antipruritic effects of luteolin from Perilla (*P. frutescens* L.) leaves. *Molecules*, 19(6), 6941-6951.

- Junghanns, J.U. and Müller, R.H. (2008). Nanocrystal technology, drug delivery and clinical applications. *International Journal of Nanomedicine*, 3(3), 295.
- Kakran, M., Shegokar, R., Sahoo, N.G, Al Shaal, L., Li, L., Müller, R.H. (2012). Fabrication of quercetin nanocrystals: comparison of different methods. *European Journal of Pharmaceutics and Biopharmaceutics*, 80(1), 113-121.
- Kandioler-Eckersberger, D., Ludwig, C., Rudas, M., Kappel, S., Janschek, E., Wenzel, C.... (2000). TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. *Clinical Cancer Research*, 6(1), 50-56.
- Kang, K.P., Park, S.K., Kim, D.H., Sung, M.J., Jung, Y.J., Lee, A.S.... (2011). Luteolin ameliorates cisplatin-induced acute kidney injury in mice by regulation of p53-dependent renal tubular apoptosis. *Nephrology Dialysis Transplantation*, 26(3), 814-822.
- Kannan, M.M. and Quine, S.D. (2011). Ellagic acid ameliorates isoproterenol induced oxidative stress: Evidence from electrocardiological, biochemical and histological study. *European Journal of Pharmacology*, 659(1), 45-52.
- Keck, C.M. and Müller, R.H. (2006). Drug nanocrystals of poorly soluble drugs produced by high pressure homogenisation. *European Journal of Pharmaceutics and Biopharmaceutics*, 62(1), 3-16.
- Kehrer, J.P. and Biswal, S.S. (2000). The molecular effects of acrolein. *Toxicological Sciences*, 57(1), 6-15.
- Khnu, U. (1976). The flavonoids: a class of semi-essential food components their role in human. *World Review of Nutrition and Dietetics*, 24, 117-191.
- Kim, D.H., Jung, E.A., Sohng, I.S., Han, J.A., Kim, T.H., Han, M.J. (1998). Intestinal bacterial metabolism of flavonoids and its relation to some biological activities. *Archives of Pharmacal Research*, 21(1), 17-23.
- Kim, H. G., Yoon, D. H., Lee, W. H., Han, S. K., Shrestha, B., Kim, C. H.... (2007). *Phellinus linteus* inhibits inflammatory mediators by suppressing redox-based NF- $\kappa$ B and MAPKs activation in lipopolysaccharide-induced RAW 264.7 macrophage. *Journal of Ethnopharmacology*, 114(3), 307-315.
- Kiuchi, H., Takao, T., Yamamoto, K., Nakayama, J., Miyagawa, Y., Tsujimura, A.... (2009). Sesquiterpene lactone parthenolide ameliorates bladder inflammation and bladder overactivity in cyclophosphamide induced rat cystitis model by inhibiting nuclear factor- $\kappa$ B phosphorylation. *The Journal of Urology*, 181(5), 2339-2348.

- Ko, L.J. and Prives, C. (1996). p53: puzzle and paradigm. *Genes and Development*, 10(9), 1054-1072.
- Kommuru, T., Gurley, B., Khan, M., and Reddy, I. (2001). Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q 10: formulation development and bioavailability assessment. *International Journal of Pharmaceutics*, 212(2), 233-246.
- Korkmaz, A., Topal, T., Oter, S. (2007). Pathophysiological aspects of cyclophosphamide and ifosfamide induced hemorrhagic cystitis; implication of reactive oxygen and nitrogen species as well as PARP activation. *Cell Biology and Toxicology*, 23(5), 303-312.
- Korkmaz, A. Oter, S., Seyrek, M., Topal, T. (2009). Molecular, genetic and epigenetic pathways of peroxynitrite-induced cellular toxicity. *Interdisciplinary Toxicology*, 2(4), 219-228.
- Korkmaz, A., Ozturk, M., Yildirim, I. (2012). Hemorrhagic cystitis; an old story with new advancements. *Journal of Experimental and Integrative Medicine*, 2(2), 93-94.
- Kotanidou, A., Xagorari, A., Bagli, E., Kitsanta, P., Fotsis, T., Papapetropoulos, A.... (2002). Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. *American Journal of Respiratory and Critical Care Medicine*, 165(6), 818-823.
- Kumar, S. and Pandey, A.K. (2013). Chemistry and biological activities of flavonoids: an overview. *The Scientific World Journal*, (2013), 1-16.
- Kwon, Y. (2001). Handbook of essential pharmacokinetics, pharmacodynamics and drug metabolism for industrial scientists: Springer Science & Business Media.
- Lakshmi, P. and Kumar, G.A. (2010). Nanosuspension technology: A review. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2(4), 35-40.
- Lamy, S., Bedard, V., Labbe, D., Sartelet, H., Barthomeuf, C., Gingras, D.... (2008). The dietary flavones apigenin and luteolin impair smooth muscle cell migration and VEGF expression through inhibition of PDGFR-beta phosphorylation. *Cancer Prevention Research (Phila)*, 1(6), 452-459.
- Lee, J.H., Park, K.H., Lee, M.H., Kim, H.T., Seo, W.D., Kim, J.Y.... (2013). Identification, characterisation, and quantification of phenolic compounds in the antioxidant activity-containing fraction from the seeds of Korean perilla (*Perilla frutescens*) cultivars. *Food Chemistry*, 136(2), 843-852.

- Lee, K.G., Shibamoto, T., Takeoka, G.R., Lee, S.E., Kim, J.H., Park, B.S. (2003). Inhibitory effects of plant-derived flavonoids and phenolic acids on malonaldehyde formation from ethyl arachidonate. *Journal of Agricultural and Food Chemistry*, 51(24), 7203-7207.
- Lee, S., Kim, W., Moon, S.O., Sung, M.J., Kim, D.H., Kang, K.P.... (2006). Rosiglitazone ameliorates cisplatin-induced renal injury in mice. *Nephrology Dialysis Transplantation*, 21(8), 2096-2105.
- Levine, L.A. and Richie, J.P. (1989). Urological complications of cyclophosphamide. *The Journal of Urology*, 141(5), 1063-1069.
- Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. *Cell*, 88(3), 323-331.
- Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Wang, J., Melino, G. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions. *Journal of Cell Science*, 113(10), 1661-1670.
- Lewis, S.A. (2000). Everything you wanted to know about the bladder epithelium but were afraid to ask. *American Journal of Physiology-Renal Physiology*, 278(6), F867-F874.
- Li, L., Wu, X., Chen, Z., Sun, S., Ye, J., Zeng, S.... (2013). Interspecies difference of luteolin and apigenin after oral administration of *Chrysanthemum morifolium* extract and prediction of human pharmacokinetics. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 68(3), 195-200.
- Li, S.D. and Huang, L. (2008). Pharmacokinetics and biodistribution of nanoparticles. *Molecular Pharmaceutics*, 5(4), 496-504.
- Li, X.S., Wang, J.X., Shen, Z.G., Zhang, P.Y., Chen, J.F., Yun, J. (2007). Preparation of uniform prednisolone microcrystals by a controlled microprecipitation method. *International Journal of Pharmaceutics*, 342(1), 26-32.
- Liao, P.H., Hung, L.M., Chen, Y.H., Kuan, Y.H., Zhang, F.B., Lin, R.H.... (2011). Cardioprotective effects of luteolin during ischemia-reperfusion injury in rats. *Circulation Journal*, 75(2), 443-450.
- Licciardi, P.V. and Underwood, J.R. (2011). Plant-derived medicines: a novel class of immunological adjuvants. *International Immunopharmacology*, 11(3), 390-398.
- Lindahl, T. and Wood, R.D. (1999). Quality control by DNA repair. *Science*, 286(5446), 1897-1905.
- Links, M. and Lewis, C. (1999). Chemoprotectants. *Drugs*, 57(3), 293-308.



- Liu, C.W., Lin, H.W., Yang, D.J., Chen, S.Y., Tseng, J.K., Chang, T.J.... (2016). Luteolin inhibits viral-induced inflammatory response in RAW264. 7 cells via suppression of STAT1/3 dependent NF- $\kappa$ B and activation of HO-1. *Free Radical Biology and Medicine*, 95, 180-189.
- Liu, Y., Tian, X., Gou, L., Sun, L., Ling, X., Yin, X. (2013). Luteolin attenuates diabetes-associated cognitive decline in rats. *Brain Research Bulletin*, 94, 23-29.
- Liversidge, G.G. and Cundy, K.C. (1995). Particle size reduction for improvement of oral bioavailability of hydrophobic drugs: I. Absolute oral bioavailability of nanocrystalline danazol in beagle dogs. *International Journal of Pharmaceutics*, 125(1), 91-97.
- Lopez, L. (2009). Distribution and biological activities of the flavonoid luteolin. *Mini Reviews in Medicinal Chemistry*, 9(1), 31-59.
- Lopez, L., Ballester, I., Abadia-Molina, A.C., Suarez, M.D., Zarzuelo, A., Martinez-Augustin, O.... (2008). Effect of flavonoids on rat splenocytes, a structure-activity relationship study. *Biochemical Pharmacology*, 76(4), 495-506.
- Ludeman, S.M. (1999). The chemistry of the metabolites of cyclophosphamide. *Current Pharmaceutical Design*, 5, 627-644.
- Macedo Baltazar, F., Almeida, P.R.C., Távora, F., Ferreira, F.V., Schmitt, F.C.... (2008). Cyclooxygenase-2 expression on ifosfamide-induced hemorrhagic cystitis in rats. *Journal of Cancer Research and Clinical Oncology*, 134(1), 19-27.
- Macedo, F.Y., Mourão, L.T., Palheta Jr, R.C., Jucá, D.M., Lima Jr, R.C., José de Sá, C.N.... (2011). Cyclooxygenase-2 contributes to functional changes seen on experimental hemorrhagic cystitis induced by ifosfamide in rat urinary bladder. *Cancer Chemotherapy and Pharmacology*, 67(4), 935-943.
- Macedo, F.Y.B., Mourão, L.T.C., Freitas, H.C., Lima-Júnior, R.C., Wong, D.V.T., Oriá, R.B.... (2012). Interleukin-4 modulates the inflammatory response in ifosfamide-induced hemorrhagic cystitis. *Inflammation*, 35(1), 297-307.
- Madhesh, M. and Vaiyapuri, M. (2012). Effect of luteolin on lipid peroxidation and antioxidants in acute and chronic periods of isoproterenol induced myocardial infarction in rats. *Journal of Acute Medicine*, 2(3), 70-76.
- Magrone, T. and Jirillo, E. (2010). Polyphenols from red wine are potent modulators of innate and adaptive immune responsiveness. *Proceedings of the Nutrition Society*, 69(3), 279-285.

- Majumdar, D., Jung, K.H., Zhang, H., Nannapaneni, S., Wang, X., Amin, A.R.... (2014). Luteolin nanoparticle in chemoprevention: in vitro and in vivo anticancer activity. *Cancer Prevention Research (Phila)*, 7(1), 65-73.
- Mandalari, G., Bisignano, C., Filocamo, A., Chessa, S., Sarò, M., Torre, G.... (2013). Bioaccessibility of pistachio polyphenols, xanthophylls, and tocopherols during simulated human digestion. *Nutrition*, 29(1), 338-344.
- Manikandan, R., Kumar, S. and Dorairajan, L.N. (2010). Hemorrhagic cystitis: a challenge to the urologist. *Indian Journal of Urology*, 26(2), 159.
- Manju, V., Balasubramanian, V., Nalini, N. (2005). Rat colonic lipid peroxidation and antioxidant status: the effects of dietary luteolin on 1,2-dimethylhydrazine challenge. *Cellular and Molecular Biology Letters*, 10(3), 535-551.
- Mant, C.T. and Hodges, R.S. (1996). Analysis of peptides by high-performance liquid chromatography. *Methods in Enzymology*, 271, 3-50.
- Marinello, A., Bansal, S., Paul, B., Koser, P., Love, J., Struck, R.... (1984). Metabolism and binding of cyclophosphamide and its metabolite acrolein to rat hepatic microsomal cytochrome P-450. *Cancer Research*, 44(10), 4615-4621.
- Mauludin, R., Müller, R.H. and Keck, C.M. (2009). Development of an oral rutin nanocrystal formulation. *International Journal of Pharmaceutics*, 370(1), 202-209.
- McCarroll, N., Keshava, N., Cimino, M., Chu, M., Dearfield, K., Keshava, C.... (2008). An evaluation of the mode of action framework for mutagenic carcinogens case study: Cyclophosphamide. *Environmental Molecular Mutagen*, 49(2), 117-131.
- McDonald, G. and Frieze, D. (2008). A problem-oriented approach to liver disease in oncology patients. *Gut*, 57(7), 987-1003.
- McNaught, A.D. (1997). Compendium of chemical terminology (Vol. 1669): Blackwell Science Oxford.
- Meng, L., Lozano, Y.F., Gaydou, E.M., Li, B. (2009). Antioxidant activities of polyphenols extracted from *Perilla frutescens* varieties. *Molecules*, 14(1), 133-140.
- Meotti, F.C., Forner, S., Lima-Garcia, J.F., Viana, A.F., Calixto, J.B. (2013). Antagonism of the transient receptor potential ankyrin 1 (TRPA1) attenuates hyperalgesia and urinary bladder overactivity in

cyclophosphamide-induced haemorrhagic cystitis. *Chemico-Biological Interactions*, 203(2), 440-447.

Merisko, L. and Liversidge, G.G. (2008). Drug nanoparticles: formulating poorly water-soluble compounds. *Toxicologic Pathology*, 36(1), 43-48.

Merisko, L. and Liversidge, G.G. (2011). Nanosizing for oral and parenteral drug delivery: a perspective on formulating poorly-water soluble compounds using wet media milling technology. *Advanced Drug Delivery Reviews*, 63(6), 427-440.

Merwid Trocha, M., Chlebda, E., Sozański, T., Magdalan, J., Książczyńska, D.... (2011). The effects of morin, a naturally occurring flavonoid, on cyclophosphamide-induced toxicity in rats. *Advances in Clinical and Experimental Medicine*, 20(6), 683-690.

Middleton, Jr., E. (1996). Biological properties of plant flavonoids: an overview. *International Journal of Pharmacognosy*, 34(5), 344-348.

Mishra, P.R., Al Shaal, L., Müller, R.H., Keck, C.M. (2009). Production and characterization of Hesperetin nanosuspensions for dermal delivery. *International Journal of Pharmaceutics*, 371(1), 182-189.

Mohamedali, K., Kedar, D., Sweeney, P., Kamat, A., Davis, D.W., Eve, B.Y.... (2005). The vascular-targeting fusion toxin VEGF 121/rGel inhibits the growth of orthotopic human bladder carcinoma tumors. *Neoplasia*, 7(10), 912-920.

Molnár, Z., Virág, E., Ordog, V. (2011). Natural substances in tissue culture media of higher plants. *Acta Biologica Szegediensis*, 55(1), 123-127.

Momoh, M.A., Muhamed, U., Agboke, A.A., Akpabio, E.I., Osonwa, U.E. (2012). Immunological effect of aqueous extract of *Vernonia amygdalina* and a known immune booster called immunace® and their admixtures on HIV/AIDS clients: a comparative study. *Asian Pacific Journal of Tropical Biomedicine*, 2(3), 181-184.

Möschwitzer, J. and Müller, R.H. (2006). New method for the effective production of ultrafine drug nanocrystals. *Journal of Nanoscience and Nanotechnology*, 6(9-10), 3145-3153.

Motawi, T.M., Sadik, N.A., Refaat, A. (2010). Cytoprotective effects of DL-alpha-lipoic acid or squalene on cyclophosphamide-induced oxidative injury: An experimental study on rat myocardium, testicles and urinary bladder. *Food and Chemical Toxicology*, 48(8), 2326-2336.

Moynihan, H. and Crean, A. (2009). *Physicochemical Basis of Pharmaceuticals*: Oxford University Press.



- Muller, B. and Muller, R. (1984). Particle size analysis of latex suspensions and microemulsions by photon correlation spectroscopy. *Journal of Pharmaceutical Sciences*, 73(7), 915-918.
- Müller, R., Jacobs, C., Kayser, O. (2001). Nanosuspensions as particulate drug formulations in therapy: rationale for development and what we can expect for the future. *Advanced Drug Delivery Reviews*, 47(1), 3-19.
- Müller, R., Jacobs, C., Kayser, O. (2002). DissoCubes - a novel formulation for poorly soluble and poorly bioavailable drugs. *Rathbone M, Hadgraft J, Roberts M. Modified-Release Drug Delivery Technology. Informa Healthcare*, 135-149.
- Müller, R.H., Gohla, S., Keck, C.M. (2011). State of the art of nanocrystals - Special features, production, nanotoxicology aspects and intracellular delivery. *European Journal of Pharmaceutics and Biopharmaceutics*, 78(1), 1-9.
- Muller, R.H. and Keck, C.M. (2004). Challenges and solutions for the delivery of biotech drugs-a review of drug nanocrystal technology and lipid nanoparticles. *Journal of Biotechnology*, 113(1), 151-170.
- Müller, R.H. and Peters, K. (1998). Nanosuspensions for the formulation of poorly soluble drugs: I. Preparation by a size-reduction technique. *International Journal of Pharmaceutics*, 160(2), 229-237.
- Murugesan, M. and Manju, V. (2013). Luteolin promotes mitochondrial protection during acute and chronic periods of isoproterenol induced myocardial infarction in rats. *The Egyptian Heart Journal*, 65(4), 319-327.
- Nekkanti, V. and Rueda, J. (2016). Nanoparticles for improved delivery of poorly soluble drugs. *Journal of Drugs*, 1(1), 18-27.
- Ness, A.R. and Powles, J.W. (1997). Fruit and vegetables, and cardiovascular disease: a review. *International Journal of Epidemiology*, 26(1), 1-13.
- Nicol, D. (2002). Cyclophosphamide and the urinary tract. *International Medical Journal*, 32(5-6), 199-201.
- Nielsen, I.L.F., Chee, W.S., Poulsen, L., Offord-Cavin, E., Rasmussen, S.E., Frederiksen, H.... (2006). Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *The Journal of Nutrition*, 136(2), 404-408.

- Niklas, K.J. and Giannasi, D.E. (1977). Flavonoids and other chemical constituents of fossil Miocene Zelkova (Ulmaceae). *Science*, 196(4292), 877-878.
- Organization, W.H. (2014). The selection and use of essential medicines: Report of the WHO expert committee, 2013 (including the 18th WHO model list of essential medicines and the 4th WHO model list of essential medicines for children) (vol. 985): World Health Organization.
- Oter, S., Korkmaz, A., Oztas, E., Yildirim, I., Topal, T., Bilgic, H. (2004). Inducible nitric oxide synthase inhibition in cyclophosphamide induced hemorrhagic cystitis in rats. *Urological Research*, 32(3), 185-189.
- Ozcan, A., Korkmaz, A., Oter, S., Coskun, O. (2005). Contribution of flavonoid antioxidants to the preventive effect of mesna in cyclophosphamide-induced cystitis in rats. *Archives of Toxicology*, 79(8), 461-465.
- Parameswaran, N. and Patial, S. (2010). Tumor necrosis factor- $\alpha$  signaling in macrophages. *Critical Reviews™ in Eukaryotic Gene Expression*, 20(2).
- Park, C.M., Jin, K.S., Lee, Y.W., Song, Y.S. (2011). Luteolin and chicoric acid synergistically inhibited inflammatory responses via inactivation of PI3K-Akt pathway and impairment of NF- $\kappa$ B translocation in LPS stimulated RAW 264.7 cells. *European Journal of Pharmacology*, 660(2), 454-459.
- Patel, J.M. (2008). A review of potential health benefits of flavonoids.
- Patel, V.R. and Agrawal, Y. (2011). Nanosuspension: An approach to enhance solubility of drugs. *Journal of Advanced Pharmaceutical Technology and Research*, 2(2), 81.
- Patravale, V. and Kulkarni, R. (2004). Nanosuspensions: a promising drug delivery strategy. *Journal of Pharmacy and Pharmacology*, 56(7), 827-840.
- Peltonen, L. and Hirvonen, J. (2010). Pharmaceutical nanocrystals by nanomilling: critical process parameters, particle fracturing and stabilization methods. *Journal of Pharmacy and Pharmacology*, 62(11), 1569-1579.
- Peters, K., Leitzke, S., Diederichs, J., Borner, K., Hahn, H., Müller, R.... (2000). Preparation of a clofazimine nanosuspension for intravenous use and evaluation of its therapeutic efficacy in murine *Mycobacterium avium* infection. *Journal of Antimicrobial Chemotherapy*, 45(1), 77-83.

- Pezzuto, J.M. (1997). Plant-derived anticancer agents. *Biochemical Pharmacology*, 53(2), 121-133.
- Philips, F.S., Sternberg, S.S., Cronin, A.P., Vidal, P.M. (1961). Cyclophosphamide and urinary bladder toxicity. *Cancer Research*, 21(11), 1577-1589.
- Pietta, P.G. (2000). Flavonoids as antioxidants. *Journal of Natural Products*, 63(7), 1035-1042.
- Pillai, A. and Gupta, S. (2005). Antioxidant enzyme activity and lipid peroxidation in liver of female rats co-exposed to lead and cadmium: effects of vitamin E and Mn<sup>2+</sup>. *Free Radical Research*, 39(7), 707-712.
- Pratheeshkumar, P., Son, Y.O., Budhraj, A., Wang, X., Ding, S., Wang, L.... (2012). Luteolin inhibits human prostate tumor growth by suppressing vascular endothelial growth factor receptor 2-mediated angiogenesis. *PLoS One* 7.12 (2012): 279-85.
- Prochazkova, D., Bousova, I., Wilhelmova, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, 82(4), 513-523.
- Qian, L., Lu, J., Ye, Z., Wang, H., Xia, Q. (2011). Luteolin reduces cardiac dysfunctions in streptozotocin-induced diabetic rats. *Chinese Journal of Applied Physiology*, 27(4), 409-414.
- Qiusheng, Z., Yuntao, Z., Rongliang, Z., Dean, G., Changling, L. (2005). Effects of verbascoside and luteolin on oxidative damage in brain of heroin treated mice. *Pharmazie*, 60(7), 539-543.
- Rabinow, B.E. (2004). Nanosuspensions in drug delivery. *Nature Reviews Drug Discovery*, 3(9), 785-796.
- Rachmawati, H., Shaal, L.A., Müller, R.H., Keck, C.M. (2013). Development of curcumin nanocrystal: physical aspects. *Journal of Pharmaceutical Sciences*, 102(1), 204-214.
- Radis, C.D., Kahl, L.E., Baker, G.L., Wasko, M.C.M., Cash, J.M., Gallatin, A.... (1995). Effects of cyclophosphamide on the development of malignancy and on long-term survival of patients with rheumatoid arthritis a 20-year follow up study. *Arthritis and Rheumatism*, 38(8), 1120-1127.
- Ramesh, G. and Reeves, W.B. (2002). TNF- $\alpha$  mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *The Journal of Clinical Investigation*, 110(6), 835-842.
- Rao, Y.M., Kumar, M.P., Apte, S. (2008). Formulation of nanosuspensions of albendazole for oral administration. *Current Nanoscience*, 4(1), 53-58.

- Rebouças, S., Da Silva, J., Bertoni, R.S., Decker, N., dos Santos, M.S., Rossatto, R.R.... (2013). Assessment of the genotoxic and mutagenic properties of *Himatanthus articulatus* bark extracts used as phytotherapeutic drug in the Amazon. *Journal of Ethnopharmacology*, 147(2), 474-480.
- Reddy, S.P., Britto, R., Vinnakota, K., Aparna, H., Sreepathi, H.K., Thota, B.... (2008). Novel glioblastoma markers with diagnostic and prognostic value identified through transcriptome analysis. *Clinical Cancer Research*, 14(10), 2978-2987.
- Reinisch, W., Dejaco, C., Feichtenschlager, T., Haas, T., Kaser, A., Miehsler, W.... (2011). Infliximab therapy for Crohn's disease-a practical guideline: actualised consensus of the working group for chronic inflammatory bowel diseases of the Austrian Society for Gastroenterology and Hepatology. *Zeitschrift fur Gastroenterologie*, 49(4), 534-542.
- Ren, S., Yang, J.S., Kalhorn, T.F., Slattery, J.T. (1997). Oxidation of cyclophosphamide to 4-hydroxycyclophosphamide and deschloroethylcyclophosphamide in human liver microsomes. *Cancer Research*, 57(19), 4229-4235.
- Ribeiro, R., Freitas, H., Campos, M., Santos, C., Figueiredo, F., Brito, G.... (2002). Tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  mediate the production of nitric oxide involved in the pathogenesis of ifosfamide induced hemorrhagic cystitis in mice. *The Journal of Urology*, 167(5), 2229-2234.
- Ribeiro, R.A., Lima-Junior, R.C., Leite, C.A.V., Mota, J.M.S., Macedo, F.Y., Lima, M.V.... (2012). Chemotherapy-induced hemorrhagic cystitis: pathogenesis, pharmacological approaches and new insights. *Journal of Experimental and Integrative Medicine*, 2(2), 95-112.
- Riboli, E. and Norat, T. (2003). Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *The American Journal of Clinical Nutrition*, 78(3), 559-569.
- Rice-Evans, C.A. (2001). Flavonoid antioxidants. *Current Medicinal Chemistry*, 8(7), 797-807.
- Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, 20(7), 933-956.
- Riddick, T.M. (1968). Control of colloid stability through zeta potential: Vol. 1: Zeta-Meter, Incorporated.
- Robinson, J. (1996). Introduction: Semi-solid formulations of oral drug delivery. *Bulletin Technique-Gattefosse*, 11-14.

- Robinson, M.M. and Zhang, X. (2011). The world medicines situation 2011, traditional medicines: Global situation, issues and challenges. *World Health Organization, Geneva*.
- Roll, R. and Kayser, D. (1986). New perspectives in acute toxicity testing of chemicals. *Toxicological Letters Supplement*, 31, 86.
- Ross, J.A. and Kasum, C.M. (2002). Dietary flavonoids: bioavailability, metabolic effects and safety. *Annual Review of Nutrition*, 22(1), 19-34.
- Rostoka, E., Isajevs, S., Baumanė, L., Line, A., Silina, K., Dzintare, M.... (2010). Effects of lycopene, indole-3-carbinol, and luteolin on nitric oxide production and iNOS expression are organ-specific in rats. *Archives of Industrial Hygiene and Toxicology*, 61(3), 275-285.
- Saban, R., Saban, M.R., Nguyen, N.B., Lu, B., Gerard, C., Gerard, N.P.... (2000). Neurokinin-1 (NK-1) receptor is required in antigen-induced cystitis. *The American Journal of Pathology*, 156(3), 775-780.
- Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E. and Chumakov, P.M. (2005). The antioxidant function of the p53 tumor suppressor. *Nature Medicine*, 11(12), 1306-1313.
- Sahoo, N., Kakran, M., Shaal, L., Li, L., Müller, R., Pal, M.... (2011). Preparation and characterization of quercetin nanocrystals. *Journal of Pharmaceutical Sciences*, 100(6), 2379-2390.
- Samy, R.P., Gopalakrishnakone, P., Ignacimuthu, S. (2006). Anti-tumor promoting potential of luteolin against 7, 12-dimethylbenz (a) anthracene-induced mammary tumors in rats. *Chemico-Biological Interactions*, 164(1), 1-14.
- Sapsford, K.E., Tyner, K.M., Dair, B.J., Deschamps, J.R., Medintz, I.L. (2011). Analyzing nanomaterial bioconjugates: a review of current and emerging purification and characterization techniques. *Analytical Chemistry*, 83(12), 4453-4488.
- Saraf, S. (2010). Applications of novel drug delivery system for herbal formulations. *Fitoterapia*, 81(7), 680-689.
- Sarkar, D., Su, Z.Z., Lebedeva, I.V., Sauane, M., Gopalkrishnan, R.V., Valerie, K. (2002). mda-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proceedings of the National Academy of Sciences*, 99(15), 10054-10059.
- Savjani, K.T., Gajjar, A.K., Savjani, J.K. (2012). Drug solubility: importance and enhancement techniques. *ISRIN Pharmaceuticals*, 2012.



- Saxena, M., Saxena, J., Nema, R., Singh, D., Gupta, A. (2013). Phytochemistry of medicinal plants. *Journal of Pharmacognosy and Phytochemistry*, 1(6).
- Scalbert, A. and Williamson, G. (2000). Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition*, 130(8), 2073S-2085S.
- Schett, G., Coates, L.C., Ash, Z.R., Finzel, S., Conaghan, P.G. (2011). Structural damage in rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis: traditional views, novel insights gained from TNF blockade, and concepts for the future. *Arthritis Research and Therapy*, 13(Suppl 1), S4.
- Seelinger Merfort, I. and Schempp, C.M. (2008). Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. *Planta Medica*, 74(14), 1667-1677.
- Senthilkumar, S., Devaki, T., Manohar, B.M. and Babu, M.S. (2006). Effect of squalene on cyclophosphamide-induced toxicity. *Clinica Chimica Acta*, 364(1), 335-342.
- Shargel, L., Wu-Pong, S., Yu, A.B. (2007). Applied biopharmaceutics and pharmacokinetics: McGraw-Hill.
- Sharma, R.K. and Agarwal, A. (1996). Role of reactive oxygen species in male infertility. *Urology*, 48(6), 835-850.
- Sharma, V., Mishra, M., Ghosh, S., Tewari, R., Basu, A., Seth, P.... (2007). Modulation of interleukin-1 $\beta$  mediated inflammatory response in human astrocytes by flavonoids: Implications in neuroprotection. *Brain Research Bulletin*, 73(1-3), 55-63.
- Shegokar, R. and Müller, R.H. (2010). Nanocrystals: industrially feasible multifunctional formulation technology for poorly soluble actives. *International Journal of Pharmaceutics*, 399(1), 129-139.
- Shen, Q., Li, X., Li, W., Zhao, X. (2011). Enhanced intestinal absorption of daidzein by borneol/menthol eutectic mixture and microemulsion. *AAPS Pharma Science and Technology*, 12(4), 1044-1049.
- Shi, R.X., Ong, C.N., Shen, H.M. (2004). Luteolin sensitizes tumor necrosis factor-alpha-induced apoptosis in human tumor cells. *Oncogene*, 23(46), 7712-7721.
- Shimoi, K., Okada, H., Furugori, M., Goda, T., Takase, S., Suzuki, M.... (1998). Intestinal absorption of luteolin and luteolin 7-O- $\beta$ -glucoside in rats and humans. *FEBS Letters*, 438(3), 220-224.

- Siafakas, A.R. and Richardson, D.R. (2009). Growth arrest and DNA damage-45 alpha (GADD45α). *The International Journal of Biochemistry and Cell Biology*, 41(5), 986-989.
- Sladek, N. (1988). Metabolism of oxazaphosphorines. *Pharmacology and Therapeutics*, 37(3), 301-355.
- Song, J., Liu, L., Li, L., Liu, J., Song, E., Song, Y. (2014). Protective effects of lipoic acid and mesna on cyclophosphamide-induced haemorrhagic cystitis in mice. *Cell Biochemistry and Function*, 32(2), 125-132.
- Sun, D., Huang, J., Zhang, Z., Gao, H., Li, J., Shen, M.... (2012). Luteolin limits infarct size and improves cardiac function after myocardium ischemia/reperfusion injury in diabetic rats. *PLoS One*, 7(3), e33491.
- Surh, Y.J. (2008). NF-kappa B and Nrf2 as potential chemopreventive targets of some anti-inflammatory and antioxidative phytonutrients with anti-inflammatory and antioxidative activities. *Asia Pacific Journal of Clinical Nutrition*, 17(Suppl 1), 269-272.
- Tahir, N.I., Shaari, K., Abas, F., Parveez, G.K., Ishak, Z., Ramli, U.S. (2012). Characterization of apigenin and luteolin derivatives from oil palm (*Elaeis guineensis* Jacq.) leaf using LC-ESI-MS/MS. *Journal of Agricultural and Food Chemistry*, 60(45), 11201-11210.
- Tak, P.P. and Firestein, G.S. (2001). NF-κB: a key role in inflammatory diseases. *Journal of Clinical Investigation*, 107(1), 7.
- Thèze, J., Alzari, P.M., Bertoglio, J. (1996). Interleukin 2 and its receptors: recent advances and new immunological functions. *Immunology Today*, 17(10), 481-486.
- Thilakarathna, S.H. and Rupasinghe, H. (2013). Flavonoid bioavailability and attempts for bioavailability enhancement. *Nutrients*, 5(9), 3367-3387.
- Thomas, S., Rafiei, S., Maghsoodlou, S., Afzali, A. (2014). Foundations of Nanotechnology, Volume Two: Nanoelements Formation and Interaction: CRC Press.
- Thommes, M., Ely, D.R., Carvajal, M.T., Pinal, R. (2011). Improvement of the dissolution rate of poorly soluble drugs by solid crystal suspensions. *Molecular Pharmaceutics*, 8(3), 727-735.
- Todorova, V., Vanderpool, D., Blossom, S., Nwokedi, E., Hennings, L., Mrak, R.... (2009). Oral glutamine protects against cyclophosphamide-induced cardiotoxicity in experimental rats through increase of cardiac glutathione. *Nutrition*, 25(7), 812-817.



- Tolley, D. and Castro, J. (1975). Cyclophosphamide-induced cystitis of the urinary bladder of rats and its treatment. *Proceedings of the Royal Society of Medicine*, 68(3), 169.
- Topal, T., Oztas, Y., Korkmaz, A., Sadir, S., Oter, S., Coskun, O.... (2005). Melatonin ameliorates bladder damage induced by cyclophosphamide in rats. *Journal of Pineal Research*, 38(4), 272-277.
- Tozer, T.N. and Rowland, M. (2006). Introduction to pharmacokinetics and pharmacodynamics: the quantitative basis of drug therapy: Lippincott Williams & Wilkins.
- Tripathi, D. and Jena, G. (2010). Effect of melatonin on the expression of Nrf2 and NF- $\kappa$ B during cyclophosphamide-induced urinary bladder injury in rat. *Journal of Pineal Research*, 48(4), 324-331.
- Ulich, T.R., Whitcomb, L., Tang, W., Tressel, P.O.C., Tarpley, J., Eunhee, S.Y.... (1997). Keratinocyte growth factor ameliorates cyclophosphamide-induced ulcerative hemorrhagic cystitis. *Cancer Research*, 57(3), 472-475.
- Uysal, E., Yilmaz, H., Ugan, Y., Altuntas, A., Doğru, A., Kutlucan, A.... (2015). AB0162 Protective effects of caffeic acid phenethyl ester (CAPE) on cyclophosphamide induced hemorrhagic cystitis in rats. *Annals of the Rheumatic Diseases*, 74(Suppl 2), 944-945.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell biology*, 39(1), 44-84.
- Verbeek, R., Plomp, A.C., van Tol, E.A., van Noort, J.M. (2004). The flavones luteolin and apigenin inhibit in vitro antigen-specific proliferation and interferon-gamma production by murine and human autoimmune T cells. *Biochemical Pharmacology*, 68(4), 621-629.
- Vial, T., Choquet-Kastylevsky, G., Descotes, J. (2002). Adverse effects of immunotherapeutics involving the immune system. *Toxicology*, 174(1), 3-11.
- Vieira, M.M., Macedo, F.Y., Filho, J.N., Costa, A.C., Cunha, A.N., Silveira, E.R.... (2004). Ternatin, a flavonoid, prevents cyclophosphamide and ifosfamide-induced hemorrhagic cystitis in rats. *Phytotherapy Research*, 18(2), 135-141.
- Wachlin, G., Augstein, P., Schröder, D., Kuttler, B., Klötting, I., Heinke, P.... (2003). IL-1 $\beta$ , IFN- $\gamma$  and TNF- $\alpha$  increase vulnerability of pancreatic beta cells to autoimmune destruction. *Journal of Autoimmunity*, 20(4), 303-312.

- Wajant, H., Pfizenmaier, K., Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death and Differentiation*, 10(1), 45-65.
- Wang,X., Wang, Q., Morris, M.E. (2008). Pharmacokinetic interaction between the flavonoid luteolin and  $\gamma$ -hydroxybutyrate in rats: potential involvement of monocarboxylate transporters. *The AAPS Journal*, 10(1), 47-55.
- Wang, X., Ouyang, Y., Liu, J., Zhu, M., Zhao, G., Bao, W.... (2014). Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. *British Medical Journal*, 29 (349) 41-49.
- Wang, Z. (2000). Transmission electron microscopy and spectroscopy of nanoparticles. Characterization of nanophase materials: Wiley-VCH Verlag GmbH.
- Watson, N.A. and Notley, R.G. (1973). Urological complications of cyclophosphamide. *British Journal of Urology*, 45(6), 606-609.
- Wei, Q., Dong, G., Yang, T., Megyesi, J., Price, P.M., Dong, Z. (2007). Activation and involvement of p53 in cisplatin-induced nephrotoxicity. *American Journal of Physiology-Renal Physiology*, 293(4), F1282-F1291.
- Williamson, G., Day, A., Plumb, G. and Couteau, D. (2000). Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transactions*, 28(2), 16-21.
- Winkel, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant physiology*, 126(2), 485-493.
- Wu, L., Zhang, J. and Watanabe, W. (2011). Physical and chemical stability of drug nanoparticles. *Advanced Drug Delivery Reviews*, 63(6), 456-469.
- Xu, K., Liu, B., Ma, Y., Du, J., Li, G., Gao, H.... (2009). Physicochemical properties and antioxidant activities of luteolin-phospholipid complex. *Molecules*, 14(9), 3486-3493.
- Yin, F., Bruemmer, D., Blaschke, F., Hsueh, W.A., Law, R.E., van Herle, A.J. (2004). Signaling pathways involved in induction of GADD45 gene expression and apoptosis by troglitazone in human MCF-7 breast carcinoma cells. *Oncogene*, 23(26), 4614-4623.
- Youan, B.B.C., Hussain, A., Nguyen, N.T. (2003). Evaluation of sucrose esters as alternative surfactants in microencapsulation of proteins by

the solvent evaporation method. *Aaps Pharmaceutical Sciences*, 5(2), 123-131.

Yu, Y., Kovacevic, Z., Richardson, D.R. (2007). Tuning cell cycle regulation with an iron key. *Cell Cycle*, 6(16), 1982-1994.

Zandvoort, A., Lodewijk, M.E., Klok, P.A., Dammers, P.M., Kroese, F.G., Timens, W. (2001). Slow recovery of follicular B cells and marginal zone B cells after chemotherapy: implications for humoral immunity. *Clinical and Experimental Immunology*, 124(2), 172-179.

Zarei, M. and Shivanandappa, T. (2013). Amelioration of cyclophosphamide-induced hepatotoxicity by the root extract of *Decalepis hamiltonii* in mice. *Food and Chemical Toxicology*, 57(0), 179-184.

Zerbini, L.F., Wang, Y., Correa, R.G., Cho, J.Y., Libermann, T.A. (2005). Blockage of NF- $\kappa$ B induces serine 15 phosphorylation of mutant p53 by JNK kinase in prostate cancer cells. *Cell Cycle*, 4(9), 1247-1253.

Zhan, Q., Chen, I.T., Antinore, M.J., Fornace, A.J. (1998). Tumor suppressor p53 can participate in transcriptional induction of the GADD45 promoter in the absence of direct DNA binding. *Molecular and Cellular Biology*, 18(5), 2768-2778.

Zhang, D., Tan, T., Gao, L., Zhao, W., Wang, P. (2007). Preparation of azithromycin nanosuspensions by high pressure homogenization and its physicochemical characteristics studies. *Drug Development and Industrial Pharmacy*, 33(5), 569-575.

Zhang, Y., Chen, X., Cheng, J., Jin, C., Zhang, Y. (2014). Correction: The reduction effect of dietary flavone C-and O-glycosides on the formation of acrylamide and its correlation and prediction with the antioxidant activity of Maillard reaction products. *RSC Advances*, 4(97), 54199-54199.

Zhang, Y. and Dong, C. (2007). Regulatory mechanisms of mitogen-activated kinase signaling. *Cellular and Molecular Life Sciences*, 64(21), 2771-2789.

## 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	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Moribund	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Convulsions	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Tremor	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Straubs Tail	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Locomotor Activity	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Abnormal Gait	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Streotypic Reaction	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Piloerection	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Writhing	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Tachypnea	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Dyspnea	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Urination	✓	x	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Feces	x	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Alopecia	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Bleeding	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x

## 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	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Nasal Discharge	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Salivation	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Lacrimation	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Exophthalmos	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Ptosis	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Loss of Corneal Reflex	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Loss of Righting Reflex	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Loss of Grasping reflex	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Diarrhea	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Analgesia	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Others	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Signature																		

Note - ✓/x: Present/Absent

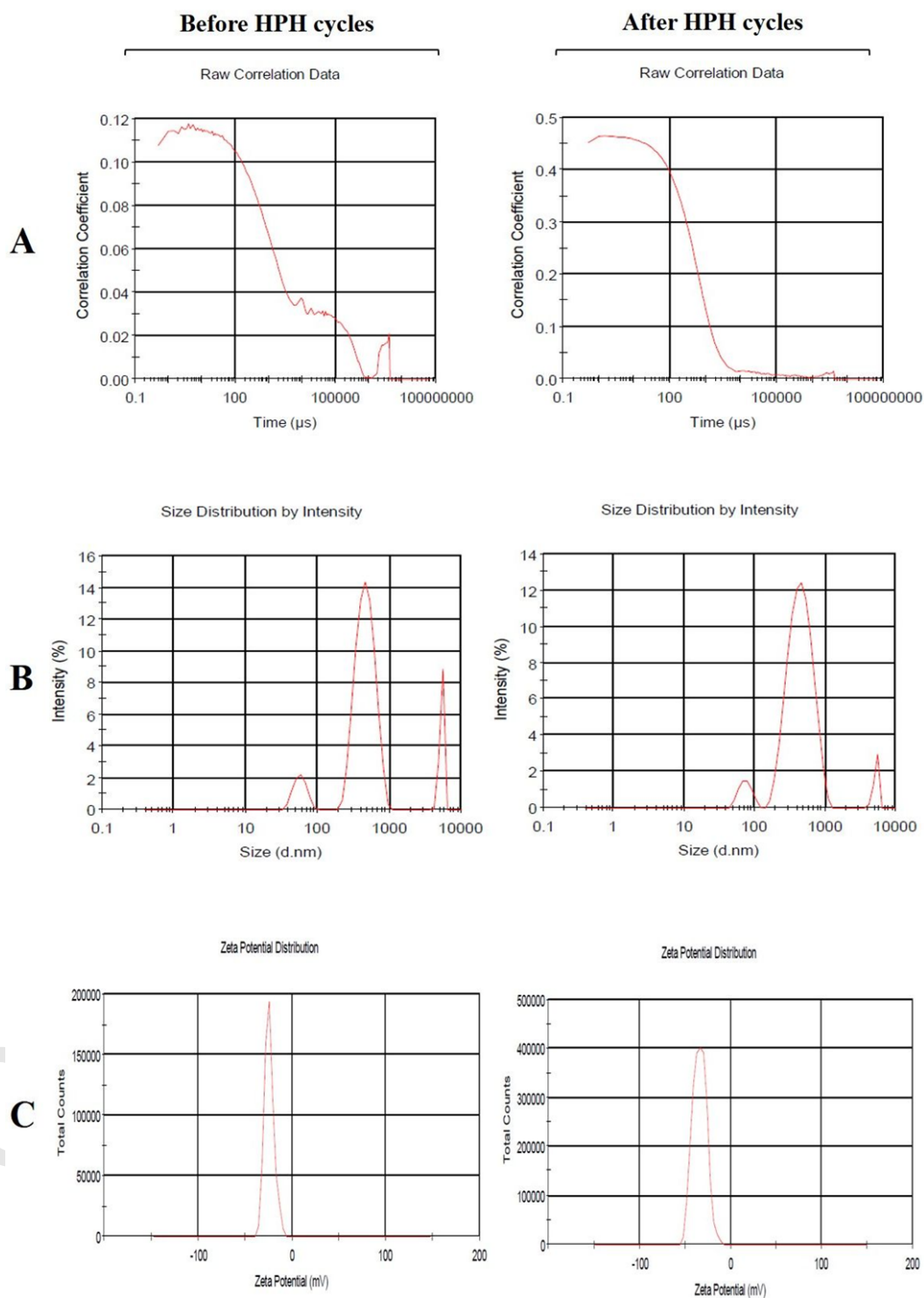
Name & Signature of Investigator:

### 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 CANSOLOR (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

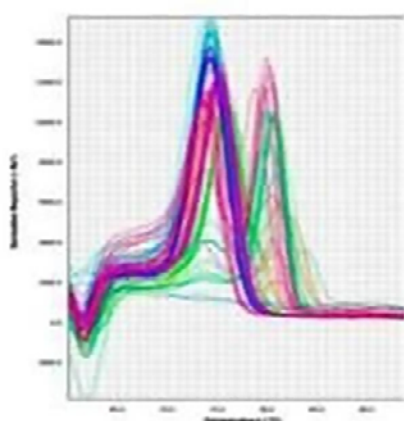
Duration: 1<sup>st</sup> August, 2013 – 31<sup>st</sup> July, 2014

(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  
Pejabat Timbalan Naib Canselor (P&I) ☎ 603-8947 1293 ☎ 603-8945 1646, Pejabat Pentadbiran TNCPI ☎ 603-8947 1608 ☎ 603-8945 1673,  
Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) ☎ 603-8947 1601 ☎ 603-8945 1596, Pejabat Pengarah, Putra Science Park (PSP)  
☎ 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 ■ VEGF ■ 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



This report generated by Rotor-Gene 6000 Series Software 1.7 (Build 87)  
Copyright ©2000-2006 Corbett Research, a Division of Corbett Life Science. All rights reserved.  
ISO 9001:2000 (Reg. No. QEC21313)

## LIST OF PUBLICATIONS

### Awards and Achievements

**Bronze medal - DIAPLAST** topical formulation containing luteolin won Bronze medal at IDEX-2015 Malaysia (Invention, Innovation & Design Expo) UiTM Puncak Alam. Nur Intan Saidah, 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, Senthil 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.



**UNIVERSITI PUTRA MALAYSIA**

**STATUS CONFIRMATION FOR THESIS / PROJECT REPORT AND COPYRIGHT**

**ACADEMIC SESSION :** \_\_\_\_\_

**TITLE OF THESIS / PROJECT REPORT :**

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

**NAME OF STUDENT:** RAVINDRAN PILLAI HARIKUMAR

I acknowledge that the copyright and other intellectual property in the thesis/project report belonged to Universiti Putra Malaysia and I agree to allow this thesis/project report to be placed at the library under the following terms:

1. This thesis/project report is the property of Universiti Putra Malaysia.
2. The library of Universiti Putra Malaysia has the right to make copies for educational purposes only.
3. The library of Universiti Putra Malaysia is allowed to make copies of this thesis for academic exchange.

I declare that this thesis is classified as :

\*Please tick (✓)

☐

**CONFIDENTIAL**

(Contain confidential information under Official Secret Act 1972).

☐

**RESTRICTED**

(Contains restricted information as specified by the organization/institution where research was done).

☐

**OPEN ACCESS**

I agree that my thesis/project report to be published as hard copy or online open access.

This thesis is submitted for :

☐

**PATENT**

Embargo from \_\_\_\_\_ until \_\_\_\_\_  
(date) (date)

**Approved by:**

\_\_\_\_\_  
(Signature of Student)  
New IC No/ Passport No.:

Date :

\_\_\_\_\_  
(Signature of Chairman of Supervisory Committee)  
Name:

Date :

**[Note : If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization/institution with period and reasons for confidentially or restricted. ]**