CYTOTOXIC, ANTIOXIDATIVE AND ANTI-HUMAN PAPILLOMAVIRUS (HPV) ACTIVITIES OF SELECTED ALLIUM SPP.

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DISSERTATION SUBMITTED TO THE INSTITUTE OF BIOLOGICAL SCIENCES, FACULTY OF SCIENCE, UNIVERSITY OF MALAYA IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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CYTOTOXIC, ANTIOXIDATIVE AND ANTI-HUMAN PAPILLOMAVIRUS (HPV) ACTIVITIES OF SELECTED ALLIUM SPP.

ABSTRACT

At the present time, the *Allium* genus consists of about 1,250 species, making it one of the largest plant genera in the world. Each member of the *Allium* genus is distinct in their appearance, colour and taste but is similarly close in their biochemical, phytochemical and nutraceutical content. This genus is revered to possess health benefiting properties and many studies have ascertained that *Alliums* play an important role in the prevention and treatment of cardiovascular disease, infectious diseases and cancers. Although *Alliums* are widely distributed and commonly used as spices and cultivated for therapeutic purposes, limited or no comprehensive data was available on their cytotoxic, antioxidant and anti-human papillomavirus (HPV) activities of its members. Hence, the present study was taken to screen and evaluate the cytotoxic, antioxidative and anti-HPV activities of 21 crude petroleum ether, chloroform and methanol extracts of selected *Alliums* from the local market, namely *Allium cepa* L., *Allium fistulosum* L., *A. porrum* L., *A. sativum* L., *A. ursinum* L. and two variants of *A. tuberosum* Rottl. (i.e. bunga kuchai and daun kuchai).

In the cytotoxicity study, the normal human lung cell line (MRC-5), human epidermal carcinoma of cervix HPV type-16 contained-cell line (CaSki) and human colon carcinoma cell line (HCT-116) were used to screen for the cytotoxic activities of the selected *Allium* spp. using the Neutral Red (NR) assay. This assay is fast, simple, accurate and sensitive in screening the toxicity of crude extracts. Crude extracts that registered effective inhibition concentration to suppress 50% cell growth in cultures
(IC$_{50}$) of 20.0 μg/ml or less are considered as active. All crude extracts were not active against the three cell lines; however three crude extracts managed to register an IC$_{50}$ value.

The antioxidative potentials of the crude Allium extracts were comprehensively evaluated using four different antioxidant bioassays namely the β-carotene bleaching inhibition assay, ferric thiocyanate (FTC) assay, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay and the reducing power assay. The total phenolic and flavonoid contents of the selected Allium spp. were also determined. The β-carotene bleaching inhibition assay and FTC assay were used to measure the inhibition of lipid peroxidation (LPO) and initial production of peroxides of the crude Allium extracts respectively. The DPPH radical scavenging assay was used to investigate the free radical scavenging ability of the crude extracts. The reduction of ferric ions by the crude extracts was tested using the reducing power assay. The Folin-Ciocalteau reagent and the aluminium chloride (AlCl$_3$) colorimetric assay were respectively used to determine the total phenolic and flavonoid contents of the crude extracts.

Overall, the petroleum ether extracts of the Alliums studied were most active in inhibiting LPO while the chloroform extracts of Alliums were more effective in inhibiting initial production of peroxides. The petroleum ether extracts of A. tuberosum Rottl. (daun kuchai) and chloroform extracts of both A. cepa L., and A. ursinum L. effectively scavenged DPPH radicals by registering inhibition concentration that causes a 50% decrease in the initial amount of free radicals (IC$_{50}$) of 3.89 mg/ml, 4.20 mg/ml and 4.55 mg/ml respectively. The methanol extracts of Allium spp. were better reducing
agents when compared to its petroleum ether and chloroform counterparts. Chloroform extracts of *A. cepa* L. possessed the highest total phenolic content of 820±0.00 mg GAEs/100 g sample while the methanol extracts of *A. porrum* L. demonstrated the highest total flavonoid content at 56.48±0.00 mg QEs/100 g sample.

Based on the persistent fact that HPV type 16 is the main causal factor of cervical cancer, and that the principal gene that is responsible for HPV replication is E6, the present study attempts to evaluate the anti-HPV type 16 E6 oncoprotein activities of the selected *Allium* spp. against CaSki cells using the simple and rapid immunocytochemistry (ICC) technique. The results showed that 20 of the *Allium* extracts was tested positive of anti-HPV type 16 E6 oncoprotein activities. The results also indicated that the methanol extracts of *A. porrum* L. induced the expression of E6 oncoprotein with increasing concentrations of extracts used.

In summary, the crude extracts of *Alliums* were not active against MRC-5, CaSki and HCT-116 cell lines. However, the extracts demonstrated promising antioxidative and anti-HPV type 16 E6 oncoprotein activities. Hence, the consumption of *Allium* spp. can be promoted for its potential in cancer and HPV infection chemoprevention.
AKTIVITI SITOTOKSIK, ANTIOKSIDAN DAN ANTI-VIRUS PAPILLOMA MANUSIA (HPV) DALAM SPESIES ALLIUM YANG TERPILIH

ABSTRAK


Esei Neutral Red (NR) telah digunakan untuk mengkaji aktiviti sitotoksik ekstrak-ekstrak mentah *Allium* ke atas tiga jenis sel terbitan manusia iaitu sel terbitan peparu normal manusia (MRC-5), sel terbitan epitelia kanser serviks manusia kandungan HPV jenis 16 (CaSkï) dan sel terbitan kanser kolon manusia (HCT-116). Ciri-ciri esei ini yang cepat, ringkas, tepat dan sensitif menjadikannya sesuai digunakan untuk membuat
penyaringan aktiviti sitotoksik *Allium* dalam kajian ini. Ekstrak mentah yang memberikan kepekatan yang merencat pertumbuhan sel dalam kultur sebanyak 50% (IC<sub>50</sub>) yang bernilai 20.0 μg/ml atau kurang dianggap aktif. Semua ekstrak mentah *Allium* adalah tidak aktif terhadap tiga jenis sel terbitan manusia tersebut tetapi tiga ekstrak mentah *Allium* didapati merekodkan nilai IC<sub>50</sub>.


Secara keseluruhannya, adalah didapati bahawa ekstrak petroleum eter *Allium* adalah paling aktif dalam merencat pengoksidaan asid linoleik manakala ekstrak kloroform adalah lebih baik dalam merencat pembentukan awal peroksid. Ekstrak petroleum eter *A. tuberosum* Rottl. (daun kuchai) dan ekstrak kloroform kedua-dua *A. cepa* L. dan *A. ursinum* L. masing-masing menunjukkan aktiviti pemusnahan radikal DPPH yang baik.
dengan merekodkan kepekatan perencatan yang mengurangkan 50% kandungan awal radikal bebas (IC$_{50}$) yang bernilai 3.89 mg/ml, 4.20 mg/ml dan 4.55 mg/ml. Dalam esei tenaga reduksi, adalah diperhatikan bahawa ekstrak-ekstrak metanol adalah lebih berpotensi berbanding kedua-dua jenis ekstrak yang lain. Ekstrak kloroform A. cepa L. mempunyai kandungan fenolik yang paling tinggi iaitu 820±0.00 mg GAEs/100 g sampel manakala ekstrak metanol A. porrum L. merekodkan kandungan flavonoid yang paling tinggi dengan 56.48±0.00 mg QEs/100 g sampel.

Atas dasar bahawa HPV jenis 16 merupakan faktor penyebab utama kanser serviks, dan E6 merupakan gen utama yang bertanggungjawab untuk replikasi HPV, maka aktiviti anti-HPV jenis 16 onkoprotein E6 sayuran Allium terpilih terhadap sel terbitan CaSki telah dianalisa dalam kajian ini menggunakan teknik imunositokimia (ICC) yang ringkas dan cepat. Dalam kajian ini, 20 daripada jumlah ekstrak mentah Allium telah menunjukkan aktiviti anti-HPV jenis 16 E6 onkoprotein yang positif. Keputusan juga menunjukkan bahawa ekstrak metanol A. porrum L. mendorong ekspresi onkoprotein E6 selaras dengan peningkatan kepekatan ekstrak kajian.

Secara kesimpulannya, ekstrak-ekstrak mentah Allium ini adalah tidak sitotoksik terhadap sel-sel terbitan MRC-5, CaSki dan HCT-116 disamping mendemonstrasikan aktiviti antioksidatif dan anti-HPV jenis 16 onkoprotein E6 yang menyakinkan. Oleh itu, pengambilan sayuran Allium dapat disarankan atas potensinya dalam pencegahan dan rawatan kanser dan jangkitan HPV.
First and foremost, I would like to dedicate my deepest appreciation and gratitude to both of my supervisors, Associate Professor Dr. Nurhayati Datuk Hj. Zainal Abidin and Professor Datin Dr. Norhanom Abdul Wahab for their invaluable guidance and encouragement. I would also like to extend my special thanks to Professor Dr. Phang Siew Moi for giving me the access to the light microscope in her laboratory.

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TABLE OF CONTENTS

Declaration  ii

Abstract   iii

Abstrak   vi

Acknowledgement  ix

Table of Contents  x

List of Abbreviations  xv

List of Tables  xxiii

List of Figures  xxvi

List of Plates  xxx

List of Appendixes  xxxi

CHAPTER ONE - INTRODUCTION  1

CHAPTER TWO – LITERATURE REVIEW  4

2.1 An Overview of Cancer  4

2.2 Carcinogenesis  4
  2.2.1 Stages of Carcinogenesis  5
  2.2.2 Classification of Carcinogens  7
  2.2.3 Factors of Carcinogenesis  8

2.3 Cancer Chemoprevention  11
  2.3.1 Mechanisms of Cancer Chemoprevention  15

2.4 Natural Product in Cancer Chemoprevention  18

2.5 Anti-cancer Agents  22
  2.5.1 Cytotoxic Activity  24
  2.5.2 Cytotoxicity Screening  25
### 3.4 Cell Culture

3.4.1 Cell Lines 85  
3.4.2 Reviving of Cell Lines 85  
3.4.3 Maintenance of Cell Cultures 86  
3.4.4 Sub-cultivation of Cells 86  
3.4.5 Cryopreservation of Cells 87  

### 3.5 Cytotoxicity Screening

3.5.1 Serial Dilution of Plant Extracts 88  
3.5.2 Preparation of Cytotoxic Screening Materials and Solutions 88  
3.5.3 Cell Enumeration 89  
3.5.4 Incubation of Cells with Plant Extract 90  
3.5.5 NR Assay 91  

### 3.6 Antioxidant Activity Screening

3.6.1 Preparation of Antioxidant Assay Stocks and Solutions 92  
3.6.2 Antioxidant Bioassay Methods 96  
3.6.3 Determination of Total Phenolic Content 101  
3.6.4 Determination of Total Flavonoid Content 102  

### 3.7 Anti-HPV 16 E6 Protein Testing

3.7.1 Serial Dilution of Plant Extracts 102  
3.7.2 Incubation of Cells with Plant Extracts 103  
3.7.3 Fixation of Cells onto Slides 103  
3.7.4 Detection of HPV E6 Protein 103  

### 3.8 ICC

3.8.1 Preparation of Solutions 104  
3.8.2 Rehydration 105  
3.8.3 Elimination of Endogenous Peroxidase Activity 105  
3.8.4 ICC Staining 105  

### CHAPTER FOUR - RESULTS

4.1 Extraction of Selected *Allium* spp. 107  

4.2 Cytotoxicity Screening of Selected *Allium* spp. 109  
4.2.1 *Allium* spp. against MRC-5 110  
4.2.2 *Allium* spp. against CaSki 115  
4.2.3 *Allium* spp. against HCT-116 116
4.2.4 Comparison of Cytotoxic Activities of Selected *Allium* spp. against MRC-5, CaSki and HCT-116

4.3 Antioxidant Activities of Selected *Allium* spp.
4.3.1 The β-carotene Bleaching Inhibition Assay
4.3.2 The FTC Assay
4.3.3 The DPPH Radical Scavenging Activity Assay
4.3.4 The Reducing Power Assay

4.4 Total Phenolic Content of Selected *Allium* spp.

4.5 Total Flavonoid Content of Selected *Allium* spp.

4.6 Antioxidant Activities of Selected *Allium* spp. and Their Total Phenolic and Flavonoid Contents
4.6.1 β-Carotene Bleaching Inhibition Activity and Total Phenolic and Flavonoid Contents
4.6.2 Lipid Peroxidation Inhibition Activity and Total Phenolic and Flavonoid Contents
4.6.3 DPPH Radical Scavenging Activity and Total Phenolic and Flavonoid Contents
4.6.4 Reducing Powers and Total Phenolic and Total Flavonoid Contents

4.7 Evaluation of the Anti-HPV 16 E6 Oncoprotein Activities
4.7.1 Anti-HPV Type 16 E6 Activities of Selected *Allium* spp.
4.7.2 Comparison of Anti-HPV Type 16 E6 Activities of Selected *Allium* spp.

CHAPTER FIVE – DISCUSSION

5.1 Preparation of Experimentation Materials
5.1.1 Plant Extraction
5.1.2 Cell Cultivation and Maintenance

5.2 Cytotoxicity Screening Using NR Assay

5.3 Cytotoxic Activities of Selected *Allium* spp.

5.4 Antioxidant for Cancer Prevention
5.4.1 Inhibition of Initial Oxidation as First Line Antioxidant Defence
5.4.2 Free Radical Scavenging as Second Line Antioxidant Defence
5.4.3 Inhibition of Oxidative Destruction as Third Line Antioxidant Defence
5.4.4 Total Phenolic Content of Selected *Allium* spp.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4.5 Total Flavonoid Content of Selected <em>Allium</em> spp.</td>
<td>241</td>
</tr>
<tr>
<td>5.4.6 Antioxidant Activities of Selected <em>Allium</em> spp.</td>
<td>242</td>
</tr>
<tr>
<td><strong>5.5 Evaluation of Anti-HPV Type 16 E6 Oncoprotein Activities Using ICC Technique</strong></td>
<td>245</td>
</tr>
<tr>
<td>5.5.1 Anti-HPV Type 16 E6 Oncoprotein Activities of Selected <em>Allium</em> spp.</td>
<td>252</td>
</tr>
<tr>
<td>5.5.2 Comparison of Anti-HPV Type 16 E6 Oncoprotein Activities of Selected <em>Allium</em> spp.</td>
<td>260</td>
</tr>
<tr>
<td><strong>5.6 Conclusion</strong></td>
<td>262</td>
</tr>
<tr>
<td>References</td>
<td>264</td>
</tr>
<tr>
<td>Appendixes</td>
<td>304</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>±</td>
<td>plus-minus</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
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<td>α-</td>
<td>alpha</td>
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<td>β-</td>
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<td>mm</td>
<td>millimeter</td>
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<td>µg/ml</td>
<td>microgram per milliliter</td>
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<td>mg/ml</td>
<td>milligram per milliliter</td>
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<tr>
<td>mg/L</td>
<td>milligram per litre</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>rpm</td>
<td>revolution per minute</td>
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<tr>
<td>t</td>
<td>time</td>
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<tr>
<td>v/v</td>
<td>volume by volume</td>
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<tr>
<td>w/v</td>
<td>weight by volume</td>
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<tr>
<td>A549</td>
<td>human alveolar basal epithelial cell line</td>
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<tr>
<td>ABTS</td>
<td>2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate)</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-I converting enzyme</td>
</tr>
<tr>
<td>ACSO</td>
<td>S-alk(en)yl-L-cysteine sulfoxide</td>
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<td>AGUS</td>
<td>atypical glandular cells of undetermined significance</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>AlCl₃</td>
<td>aluminium chloride</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine amino transferase</td>
</tr>
<tr>
<td>AMF</td>
<td>autocrine motility factor</td>
</tr>
<tr>
<td>ASC</td>
<td>ascorbic acid</td>
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<tr>
<td>ASCO</td>
<td>S-alk(en)yl-L-cysteine sulfoxides</td>
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<td>ASCUS</td>
<td>atypical squamous cells of undetermined significance</td>
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<tr>
<td>AST</td>
<td>aspartate amino transferase</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>B.C.</td>
<td>before century</td>
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<td>Bcl 7402</td>
<td>human hepatocellular carcinoma cell line</td>
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<td>BGC 823</td>
<td>human gastric carcinoma cell line</td>
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<tr>
<td>BHA</td>
<td>butylated hydroxyanisole</td>
</tr>
<tr>
<td>BRR</td>
<td>Briggs-Rauscher reaction</td>
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<tr>
<td>C33a</td>
<td>human cervix carcinoma cell line</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecules</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CaSki</td>
<td>human epidermoid carcinoma of cervix HPV type 16-contained cell line</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CIN</td>
<td>cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>CIS</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>Cu</td>
<td>cuprum</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DAB</td>
<td>3’-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DADS</td>
<td>diallyl disulfide</td>
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<tr>
<td>DAS</td>
<td>diallylsulfide</td>
</tr>
<tr>
<td>DATS</td>
<td>diallyl trisulfide</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(a)-anthracene</td>
</tr>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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DNA: deoxyribonucleic acid
DPPH: 1,1-diphenyl-2-picrylhydrazyl hydrate
DPPH°: 1,1-diphenyl-2-picrylhydrazyl hydrate radical
e.g.: for example
E: early region
E6AP: E6-associated protein
EBV-EA: Epstein-Barr virus early antigen
EC: (-)-epicatechin
ECG: (-)-epicatechin-3-gallate
EDTA: ethylenediaminetetraacetic acid
EGC: (-)-epigallocatechin
EGCG: (-)-epigallocatechin-3-gallate
ELISA: enzyme-linked immunosorbent assay
EMEM: Eagle’s Minimum Essential Medium
EPA: eicosapentaenoic acid
ET-1: endothelin-1
ET_{AR}: endothelin A receptor
FAEs: ferulic acid equivalents
FBS: foetal bovine serum
Fe^{2+}: ferrous ion
Fe^{3+}: ferric ion
FeCl_2: ferrous chloride
FeCl_3: ferric chloride
FM: fresh matter
FRAP: ferric reducing antioxidant power
FTC: ferric thiocyanate
GAEs: gallic acid equivalents
Gps2: G-protein pathway suppressor gene
GSH: glutathione
GSH.Px: glutathione peroxidase
GSSG: glutathione disulfide
GST: glutathione-S-transferase
H₂: hydrogen
H₂O: water
H₂O₂: hydrogen peroxide
HCl: hydrochloric acid
HCT-15: human colon tumour cell line
HCT-116: human colon carcinoma cell line
HDAC: histone deacetylase
HDL: high-density lipoprotein
HeLa: human epithelial adenocarcinoma of cervix HPV type 18-contained cell line
Hep-G2: human hepatocellular carcinoma cell line
HEPES: N-2-Hydroxylethyl-Piperazine-N-2-Ethane-Sulfonic Acid
HIV: human immunodeficiency virus
HL-60: human acute promyelocytic leukemia cell line
HPV: human papillomavirus
HRP: horseradish peroxidase
HSIL: high-grade intraepithelial lesion
HT-29: human colorectal adenocarcinoma cell line
hTERT: human telomerase reverse transcriptase
i.e.: that is to say
I3C: indole-3-carbinol
IARC: International Agency for Research on Cancer
IC₅₀: concentration that causes a 50% inhibition
ICC: immunocytochemistry
IU: international unit
K: potassium
K₂HPO₄: potassium phosphate anhydrous
K₃[Fe(CN)₆]: potassium ferrocyanide
KB: human nasopharyngeal carcinoma cell line
KH₂PO₄: potassium dihydrogen orthophosphate
KPL-1: human mammary carcinoma cell line
L: late region
LDH: lactate dehydrogenase
LDL: low-density lipoprotein
LLC: Lewis lung carcinoma
LOH: lipid alcohol
LOOH: lipid hydroperoxides
LPS: lipopolysaccharide
LSAB: labelled strepavidin-biotin
LSIL: low-grade squamous intraepithelial lesion
M344: 4-dimethylamino-N-(6-hydroxycarbamoyl-hexyl)-benzamide
MALT: mucosa associated lymphoid tissue
MCF-7: human mammary adenocarcinoma cell line
MDA-MB231: human mammary adenocarcinoma cell line
MDR: multiple drug resistance-1
MKL-F: human mammary carcinoma cell line
Mn: mangan
MRC-5: normal human lung cell line
MRSA: methicillin-resistant Staphylococcus aureus
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na: sodium
NaB: sodium butyrate
NaCl: sodium chloride
NADPH: nicotinamide adenine dinucleotide phosphate
NaHCO₃: sodium bicarbonate
NaH₂PO₄: sodium dihydrogen phosphate
Na₂CO₃: sodium carbonate
Na₂HPO₄: sodium phosphate anhydrous
NBT: nitroblue tetrazolium
NCI: National Cancer Institute
NDEA: N-nitrosodiethylamine
NF-κB: nuclear factor-kappaB
NH₄SCN: ammonium thiocyanate
NK: natural killer
NO°: nitric oxide
NOO°: nitrogen peroxide
NO₂: nitrogen oxide
NO₂⁻: nitrite oxide
NR: Neutral Red
NSAID: non-steroidal anti-inflammatory drug
¹O₂: singlet oxygen
O₂: oxygen
O₂⁻: superoxide radicals
OD: optical density
OH⁺: hydroxyl radicals
ONO²°: peroxynitrite
ORAC: oxygen radical absorbance capacity
ORF: open reading frame
OSCN⁻: hypothiocyanite
P-gp: P-glycoprotein
p53: tumour suppressor gene
p97: early promoter gene
PBMC: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PC-3: human prostate cancer cell line
PDE: phosphodiesterase
PGE2: prostaglandin E2
pRb: retinoblastoma gene
PUFA: polyunsaturated fatty acids
PVA: polyvinyl alcohol
QEs: quercetin equivalents
RAW 264.7: macrophage; Abelson murine leukemia virus transformed
RIF: radiation-induced fibrosis
RNA: ribonucleic acid
RNS: reactive nitrogen species
RO₂⁺: peroxyl radicals
ROS: reactive oxygen species
RPMI: Roswell Park Memorial Institute
RS°: thiyl radical
RS°O: sulfinyl radical
RS\textsubscript{n}: polysulfide radical
RSO\textsubscript{2}°: sulfonyl radical
RSS: reactive sulfur species
SERM: selective estrogen receptor modulator
SAC: S-allylcysteine
SAMC: S-allylmercaptocysteine
Saos-2: human osteosarcoma cell line
SCN\textsuperscript{-}: thiocyanate
SD: standard deviation
SF: scatter factor
SiHa: human squamous cell carcinoma cell line
SIL: squamous intraepithelial lesions
SMCS: S-methylcysteine sulfoxide
spp.: species
SOD: superoxide dismutase
TBA: thiobarbituric acid
TCA: trichloroacetic acid
TEAC: trolox equivalent antioxidant capacity
TGF-\beta1: transforming growth factor- \beta1
TRAP: total radical-trapping antioxidant parameter
TSA: trichostatin A
TXA\textsubscript{2}: thromboxane A\textsubscript{2}
U937: human histiocytic lymphoma cell line
URR: upstream regulatory region
UV: ultraviolet
VEGF: vascular endothelial growth factor
VLDL: very low-density lipoprotein
WHO: World Health Organization
XOD: xanthine/xanthine oxidase
Zn: zinc
LIST OF TABLES

Table 2.1  Anti-cancer agents and their mechanism(s) of action
Table 2.2  The types of methods used to evaluate antioxidant activity
Table 2.3  An overview of the regions of the HPV genome
Table 2.4  The domestic *Allium* and its usages
Table 3.1  The pre-screening protocol of *Allium* spp. crude extracts in DPPH scavenging activity assay
Table 3.2  The determination of IC$_{50}$ value of *Allium* spp. crude extracts in DPPH scavenging activity assay
Table 4.1  The dry weight (g) yield of the *Allium* samples
Table 4.2  The percentage of yield of crude extracts of selected *Allium* spp.
Table 4.3  The percentage of inhibition of doxorubicin against MRC-5, CaSki and HCT-116 cell lines
Table 4.4  IC$_{50}$ value of doxorubicin against MRC-5, CaSki and HCT-116 cell lines
Table 4.5(a)  The cell killing percentage of *Allium* spp. at various concentrations of crude petroleum ether extracts
Table 4.5(b)  The cell killing percentage of *Allium* spp. at various concentrations of crude chloroform extracts
Table 4.5(c)  The cell killing percentage of *Allium* spp. at various concentrations of crude methanol extracts
Table 4.6  The IC$_{50}$ values of *Allium* spp. crude extracts tested against MRC-5, CaSki and HCT-116 cell lines
Table 4.7  The antioxidant activity of various concentrations of BHA as determined by the β-carotene bleaching inhibition assay
Table 4.8  The antioxidant activity of BHA (0.2 mg/ml) as determined by the FTC assay
Table 4.9  The anti-DPPH radical activities of crude petroleum ether extracts of *A. tuberosum* Rottl. (daun kuchai) and crude chloroform extracts of *A. cepa* L. and *A. ursinum* L.
Table 4.10 Summary of IC<sub>50</sub> values of the active crude extracts and positive references, ascorbic acid and BHA in the DPPH radical scavenging activity assay

Table 4.11 The reducing power of various concentrations of ascorbic acid

Table 4.12(a) The reducing power of various concentrations of crude petroleum ether extracts of selected *Allium* spp.

Table 4.12(b) The reducing power of various concentrations of crude chloroform extracts of selected *Allium* spp.

Table 4.12(c) The reducing power of various concentrations of crude methanol extracts of selected *Allium* spp.

Table 4.13 Total phenolic content of selected *Allium* spp. as determined using the Folin-Ciocalteau assay

Table 4.14 Total flavonoid content of selected *Allium* spp. as determined using the AlCl<sub>3</sub> colorimetric assay

Table 4.15 Antioxidant activity and total phenolic and flavonoid content status

Table 4.16 The relationship between β-carotene bleaching inhibition activities of crude *Allium* spp. extracts and their total phenolic and flavonoid contents

Table 4.17 The relationship between antioxidant activities of crude *Allium* spp. extracts determined by the FTC assay and their total phenolic and flavonoid contents

Table 4.18 The relationship between DPPH radical scavenging activities of crude *Allium* spp. extracts and their total phenolic and flavonoid contents

Table 4.19 The relationship between reducing powers of crude *Allium* spp. extracts and their total phenolic and flavonoid contents

Table 4.20 Appearances of CaSki cells treated with crude petroleum ether extracts of *Allium fistulosum* L.

Table 4.21 Appearances of CaSki cells treated with crude petroleum ether extracts of *Allium tuberosum* Rottl. (daun kuchai)

Table 4.22 Appearances of CaSki cells treated with crude petroleum ether extracts of *Allium cepa* L.

Table 4.23 Appearances of CaSki cells treated with crude chloroform extracts of *Allium sativum* L.
Table 4.24  Appearances of CaSki cells treated with crude chloroform extracts of
*Allium ursinum* L.

Table 4.25  Appearances of CaSki cells treated with crude methanol extracts of
*Allium tuberosum* Rottl. (bunga kuchai)

Table 4.26  Appearances of CaSki cells treated with crude methanol extracts of
*Allium porrum* L.
LIST OF FIGURES

Figure 2.1  The formation and depletion of ROS
Figure 2.2  ROS, oxidative stress and antioxidants
Figure 2.3(a)  First line antioxidant defence mechanism-suppression of free radical formation
Figure 2.3(b)  Second line antioxidant defence mechanism-radical scavenging and inhibition of chain initiation and propagation
Figure 2.3(c)  Third line antioxidant defence mechanism-sacrificial antioxidants and termination of oxidative stress progression
Figure 2.4  The schematic representation of the HPV genome
Figure 2.5  The brief illustration of carcinogenesis of cervical cancer
Figure 2.6  Diagram illustrating the three-step indirect labelled avidin binding (LAB) procedure
Figure 2.7  The water- and lipid-soluble organosulfur compounds in *Allium* vegetables
Figure 2.8  The formation of allicin from alliin
Figure 2.9  Chemical structure of quercetin
Figure 4.1(a)  Growth inhibition of MRC-5 cells by crude petroleum ether extracts of selected *Allium* spp. using the NR assay
Figure 4.1(b)  Growth inhibition of MRC-5 cells by crude chloroform extracts of selected *Allium* spp. using the NR assay
Figure 4.1(c)  Growth inhibition of MRC-5 cells by crude methanol extracts of selected *Allium* spp. using the NR assay
Figure 4.2(a)  Growth inhibition of CaSki cells by crude petroleum ether extracts of selected *Allium* spp. using the NR assay
Figure 4.2(b)  Growth inhibition of CaSki cells by crude chloroform extracts of selected *Allium* spp. using the NR assay
Figure 4.2(c)  Growth inhibition of CaSki cells by crude methanol extracts of selected *Allium* spp. using the NR assay
Figure 4.3(a)  Growth inhibition of HCT-116 cells by crude petroleum ether extracts of selected *Allium* spp. using the NR assay
Figure 4.3(b) Growth inhibition of HCT-116 cells by crude chloroform extracts of selected *Allium* spp. using the NR assay

Figure 4.3(c) Growth inhibition of HCT-116 cells by crude methanol extracts of selected *Allium* spp. using the NR assay

Figure 4.4(a) Antioxidative activity of crude petroleum ether extracts from selected *Allium* spp. in the β-carotene bleaching inhibition assay

Figure 4.4(b) Antioxidative activity of crude chloroform extracts from selected *Allium* spp. in the β-carotene bleaching inhibition assay

Figure 4.4(c) Antioxidative activity of crude methanol extracts from selected *Allium* spp. in the β-carotene bleaching inhibition assay

Figure 4.5(a) The inhibition of lipid oxidation by crude petroleum ether extracts of *Allium* spp. as determined by the FTC assay

Figure 4.5(b) The inhibition of lipid oxidation by crude chloroform extracts of *Allium* spp. as determined by the FTC assay

Figure 4.5(c) The inhibition of lipid oxidation by crude methanol extracts of *Allium* spp. as determined by the FTC assay

Figure 4.6 The determination of IC$_{50}$ values of ascorbic acid and BHA as standard positive references in the DPPH radical scavenging activity assay

Figure 4.7 The determination of IC$_{50}$ values of the active crude extracts of *A. cepa* L., *A. ursinum* and *A. tuberosum* Rottl. (daun kuchai) in the DPPH radical scavenging activity assay

Figure 4.8(a) Summary of the reducing power of crude petroleum ether extracts of selected *Allium* spp.

Figure 4.8(b) Summary of the reducing power of crude chloroform extracts of selected *Allium* spp.

Figure 4.8(c) Summary of the reducing power of crude methanol extracts of selected *Allium* spp.

Figure 4.9 Total phenolic content of selected *Allium* spp. expressed as mg GAEs/100 g sample

Figure 4.10 Total flavonoid content of selected *Allium* spp. expressed as mg QEs/100 g sample
Figure 4.11 Staining intensity of CaSki cells treated with *A. cepa* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.12 Staining intensity of CaSki cells treated with *A. fistulosum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.13 Staining intensity of CaSki cells treated with *A. porrum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.14 Staining intensity of CaSki cells treated with *A. sativum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.15 Staining intensity of CaSki cells treated with *A. ursinum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.16 Staining intensity of CaSki cells treated with *A. tuberosum* Rottl. (bunga kuchai). Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.17 Staining intensity of CaSki cells treated with *A. tuberosum* Rottl. (daun kuchai). Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.18(a) The summary of reddish-brown stain intensity of CaSki cells treated with crude petroleum ether extracts of selected *Allium* spp. as determined by ICC. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

Figure 4.18(b) The summary of reddish-brown stain intensity of CaSki cells treated with crude chloroform extracts of selected *Allium* spp. as determined by ICC. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)
Figure 4.18(c) The summary of reddish-brown stain intensity of CaSki cells treated with crude methanol extracts of selected *Allium* spp. as determined by ICC. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)
LIST OF PLATES

Plate 3.1  The Allium spp. selected from the Malaysian local market for investigation

Plate 4.1  The classifications of reddish-brown stain intensity that indicate the presence of E6 oncoprotein in CaSki cells after ICC

Plate 4.2(a)  Untreated CaSki cells without anti-HPV type 16 E6 monoclonal antibody incubation (40X)

Plate 4.2(b)  Untreated CaSki cells with anti-HPV type 16 E6 monoclonal antibody incubation (40X)

Plate 4.3  Appearance of viable cells and dead and/or dying cells after ICC

Plate 5.1(a)  Untreated CaSki cells without anti-HPV type 16 E6 monoclonal antibody incubation (40X)

Plate 5.1(b)  Untreated CaSki cells with anti-HPV type 16 E6 monoclonal antibody incubation (40X)
LIST OF APPENDIXES

Appendix A  Determination of Total Phenolic and Flavonoid Content of Selected *Allium* spp.
CHAPTER 1

INTRODUCTION

In 2002, 26,089 cancer cases were reported among Malaysians residing in Peninsular Malaysia and in the following year, 21,464 cases had been registered. Breast, cervix, colon, ovary and lung cancers were among the few frequent cancers affecting women in Malaysia. Cervical cancer is Malaysia’s second most common carcinoma after breast cancer, accounting for 12.0% and 12.9% respectively of all malignancies in women, in the year 2002 and 2003 (Lim et al., 2003; 2004).

Human carcinogenesis is a prolonged multistage process which involves transformation of normal cells to cancerous cells. It can be divided into three stages - initiation, promotion and progression. DNA damaging agents such as radiations, chemicals and viruses will initiate and promote carcinogenesis when cell proliferation takes place uncontrollably (Reddy et al., 2003).

Chemoprevention approaches have become one of the important strategies in controlling human cancer. Numerous researches have been done to exploit potential natural products in preventing cancers and other life threatening diseases such as cardiovascular diseases, neurodegenerative disorders, diabetes and infectious diseases. Chemoprevention can be applied at three different levels, depending on the target of intervention. In order to eradicate genetically abnormal products, chemoprevention is introduced at the cellular level. At the tissue level, chemoprevention approaches are applied to suppress the formation of pre-invasive tissues and subsequently prevent or retard carcinogenesis (Flora et al., 2001).
In recent years, plant sources have made great contribution towards cancer research. Cragg et al., (1997) reported that 60% of the approved chemotherapeutic agents and their sources were all plant-derived and 11% of the 252 drugs that were considered novel by World Health Organization (WHO) were plant-derived (Rates, 2001). The search for novel drugs in treatment of cancer, cardiovascular diseases, infectious diseases and viral diseases has become evidently important as more researches were published; describing the role of plant-derived bioactive compounds as antibiotics, anti-tumour drugs, anti-inflammatory drugs, immunomodulators, drugs in the treatment of tropical diseases and psychiatric problems (Hamburger and Hostettmann, 1991).

*Allium* is the largest genus in the widely distributed Alliaceae family. The *Alliums* are cultivated for food, medicine, garden ornamentals, and as weeds. The availability of modern scientific techniques and applications helped discover the many benefits of *Allium* vegetables. *Allium cepa* L. and *A. sativum* L. are the most commonly known members of the *Allium* genus. The present study attempts to investigate the potential of selected edible *Allium* spp. in long-term chemoprevention. Hence, seven selected *Allium* vegetables were evaluated for their cytotoxic, antioxidative and anti-human papillomavirus (HPV) type 16 E6 oncoprotein activities.

The objectives of the present study are as follows:

a) to screen cytotoxic potentials of the crude petroleum ether, chloroform and methanol extracts of seven *Alliums* against normal human lung cell line (MRC-5), human epidermal carcinoma of cervix HPV type-16 contained-cell line (CaSki) and human colon carcinoma cell line (HCT-116) using the Neutral Red (NR) assay;
b) to evaluate the antioxidative potentials of the crude petroleum ether, chloroform and methanol extracts of seven *Allium* using the β-carotene bleaching assay, ferric thiocyanate (FTC) method, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical scavenging assay and the reducing power assay;

c) to investigate the total phenolic content of the crude petroleum ether, chloroform and methanol extracts of seven *Allium* using the Folin-Ciocalteau assay;

d) to investigate the total flavonoid content of the crude petroleum ether, chloroform and methanol extracts of seven *Allium* using the aluminium chloride (AlCl₃) colorimetric assay; and

e) to evaluate the potential of the crude petroleum ether, chloroform and methanol extracts of seven *Allium* in suppressing or inhibiting the expression of HPV type 16 E6 oncoprotein in CaSki cells using the immunocytochemistry (ICC) technique.
2.1 An Overview of Cancer

Cell growth that is no longer subjected to normal cellular control mechanism leads to the formation of an abnormal group of cells which invade and destroy the surrounding tissue is known as cancer (Frenkel, 2003). In the United States of America, cancer is the second major cause of death (Jemal et al., 2005). In the year 2000, there were over ten million new incidences of cancer, with an estimated mortality rate of over six million. A 22% increase in cancer incidence and mortality has been recorded since 1990 and the cancer of lung, breast, colorectal and stomach were the most frequent cancers (Parkin et al., 2001).

Incorrect diet, genetic predisposition and unhealthy environment may contribute to cancers. It was reported that at least 32% of all the cancers worldwide were due to incorrect diet. 80% of colon cancer incidence was associated with inappropriate diet (Reddy et al., 2003). Carcinogens found in the diet such as aflatoxins, alcohol, nitrosamines, rancid fats and cooking oils, additives and preservatives may initiate the early stages of carcinogenesis. When a combination of foods as such is added to unhealthy lifestyle like smoking, lack of exercise and stress, UV radiation and free radicals, DNA damages may occur and this contributes to the progression of cancer.

2.2 Carcinogenesis

Cancer initiation and other stages of carcinogenesis are very much influenced by the mutations that occur in the somatic cells. These mutations are the very key factor leading to cancer (Flora and Ferguson, 2005). The process where initiation of a
mutation in a single gene due to the presence of physical or chemical agents that consequently causes the promotion and progression of irreversible genetic changes resulting in genetic mutation is known as carcinogenesis (Martinez et al., 2003). Carcinogenesis which is a multistage process and transformation of normal cells to cancerous cells can be divided into three stages - initiation, promotion and progression. The presence of DNA damaging agents will initiate carcinogenesis and the process is promoted when cell proliferation takes place uncontrollably. Additional genetic alterations occur during the progression stage (Greenwald, 2002).

2.2.1 Stages of Carcinogenesis
Carcinogenesis is a prolonged multistage process that is contributed by genetic damages and epigenetic changes. Animal models were once the primary source of study of carcinogenesis but in the recent decade, molecular analysis of cancer-related genes in human is used to create a more sophisticated view of carcinogenesis (Harris, 1991). Carcinogenesis have been said to include the stages of initiation, promotion and progression and these stages are separate biological processes (Bower and Waxman, 2006).

Initiation
After exposure to a carcinogen, a cell is ‘induced’ and it takes months and many cell generations to develop detectable phenotypes that indicate carcinogenicity of the cell. This is the so called initiation stage (Pitot, 2002). During initiation, the cellular DNA goes through mutation and activates the oncogenes. On the other hand, the tumour suppressor genes will be inactivated. Initiation is an irreversible stage and the single gene mutation is usually caused by genotoxic agents such as dimethylbenzanthracene (DMBA) (Bower and Waxman, 2006).
Promotion
Promotion is a subsequent process of initiation and is reversible in its early stages. During promotion, the initiated cell is repetitively exposed to promoting agent and goes through a prolonged cell proliferation process. The latent phenotype of the initiated cell is expressed through clonal expansion, transforming a single cancer cell into a mass of cancerous tissue. Usually, promoting agents are non-genotoxic agents such as hormones (Kleinsmith, 2006).

Progression
Progression is the final stage of carcinogenesis. It is irreversible and genetic damages are often found in this stage. During progression, repetitive mutations and epigenetic changes of the gene create more aberrant cells with abnormalities that characterize cancer cells. Over time, these cells become more dominant than the normal cells and acquire more aberrant traits through the repetitive process of this cycle resulting in clonal selection.

A preference for better surviving traits exists among the cells where cells exhibiting advantageous properties such as increased growth rate, increased invasiveness and more adaptable to new distant sites slowly dominate the cell population. The new cell population acts as a clone and the repetition of the process above creates another cell population with enhanced growth regulatory mechanisms. This process is known as clonal selection and this characteristic enhances the overall survival rate of the aberrant cells, the invasiveness and metastasis properties of these cells (Kleinsmith, 2006).
2.2.2 Classification of Carcinogens

Any agent that can induce or increases the risk of developing cancer is defined as carcinogen (King, 2000). Most often, human cancers are caused by exposure to carcinogens such as viruses, radiation and both natural and manmade chemical carcinogens. Carcinogens can be classified as genotoxic and non-genotoxic (Reddy et al., 2003).

Genotoxic Carcinogens

Genotoxic carcinogens cause irreversible damage to the DNA; either directly or indirectly through metabolic activation (King, 2000). The genotoxic carcinogens such as dimethylsulfate and ethylene imines are primary, direct-acting alkylating agents which react with nucleic acids thus directly affecting the cellular constituents of the cell. Another class of carcinogens is named pro-carcinogens. Pro-carcinogens induce carcinogenesis through metabolic activation and the few common members in this carcinogen class are polycyclic aromatic hydrocarbon, hydrazine, nitrosamines and a few inorganic mineral such as cadmium and plutonium (Timbrell, 2000).

Non-genotoxic Carcinogens

Non-genotoxic or epigenetic carcinogens are carcinogens that does not damage the DNA but instead act in other ways to promote cell growth (King, 2000). This class of carcinogens initiates carcinogenesis and is available in a vast range of compounds consisting of organic chemicals to metal compounds. The variation of the epigenetic carcinogens has suggested that more than one mechanism is required in carcinogenesis. The epigenetic carcinogens are categorized as promoters such as phorbol esters, hormones molecules like estrogens, solid carcinogens such as
asbestos and plastics, immunosuppressants, purine analogues and co-carcinogens.
(Timbrell, 2000).

2.2.3 Factors of Carcinogenesis

The cancer-causing factors can be divided into genetic and environmental factors. Genetic factors involve germ-line genetic mutations while environmental factors are sub-divided into physical, chemical and biological factors.

Genetic Factors

The germ-line genetic mutations can be inherited or may be sporadic incidence. Germ-line genetic mutations in oncogenes and tumour suppressor genes may contribute to the initiation of carcinogenesis. Oncogenes are genes that cause cancer while in contrast; tumour suppressor genes are genes that suppress cancer by interrupting the cell cycle and also cell mutations and proliferations that contribute to cancer (Bower and Waxman, 2006).

For instance, it was reported that in lung cancers, there is an over-expression of 50-90% of Bcl-2 oncogene, which is involved in the regulation of apoptosis. The over-expression of Bcl-2 may prevent the release of cytochrome-c, an intermediate in apoptosis. As such, apoptosis induced by ultraviolet irradiation, cytotoxic agents, p53 and e-myc is hindered, resulting in an increased risk of lung carcinogenesis (Wan et al., 2006).

On the other hand, the genetic alteration of the tumour suppressor gene, p53 was known to be directly linked with the occurrences of cancers. In fact, it was shown that mutation of p53 was involved in approximately 50% of non-small cell lung
carcinoma and 90% in small cell lung carcinoma (Rodin and Rodin, 2005; Viktorsson et al., 2005). The p53 gene is important in triggering cell cycle arrest and activation of apoptosis (Lane and Hupp, 2003).

Environmental Factors

The environmental factors are sub-divided into physical, chemical and biological carcinogens. Cancers caused by these carcinogens are avoidable because these carcinogens are amenable to chemoprevention strategies (Martin-Moreno et al., 2008).

i) Physical Carcinogen

The major physical carcinogen is radiation. The source of radiation is found everywhere and it can be both ionizing and non-ionizing radiation sources. The ionizing sources include γ-rays, α-particles from radon and X-rays while the non-ionizing sources are ultraviolet (UV) light from the sun, electromagnetic fields, ultrasound imaging and microwave and radio frequency radiation from electrical devices (Bower and Waxman, 2006). O’ Sullivan and Levin, (2003) reported that long-term exposure to ionizing radiation therapy will increase the risk of subcutaneous radiation-induced fibrosis (RIF) while sunlight exposure results in the mutation of p53 gene causing UV radiation-induced neoplasm (Ravanat et al., 2001).

ii) Chemical Carcinogen

Chemical carcinogenesis is a multi-stage process that begins with initiation, promotion and progression of a carcinoma induced by a chemical carcinogen. A single exposure to a carcinogen may be sufficient to cause genetic alterations and
induce carcinogenesis. Carcinogenesis is promoted with the repetitive exposure to a promoter in multiple doses followed by the progression of carcinogenesis. The promotion stage is reversible but however, both of the initiation and progression stage are irreversible (King, 2000).

Chemical carcinogens may function at any or all of these stages and may be compounds can be easily sourced from our diet. It was estimated that approximately 30% of all cancer mortalities are caused by what we eat (Willett, 2006). For instance, high alcohol consumption has been related with the increased onset of oral, oesophageal, pharynx, larynx as well as breast and liver cancers (Boffetta et al., 2006) while tobacco smoking superimposes the risk of lung carcinogenesis (Li et al., 2008a). The intake of red and processed meat should be reduced or avoided as it may increase the risk of colon cancer (Martin-Moreno et al., 2008).

iii) Biological Carcinogen

Approximately 8% of cancer incidence globally is contributed by infectious agents such as viruses, bacteria or parasites (Parkin, 2006). For example, cervical cancer is often associated with the infection of HPV (Rocha-Zavaleta et al., 2004) and it is confirmed that HPV DNA is detected in over 95% of invasive cervical carcinomas (De Francesco et al., 2005). Other viruses associated with cancer are hepatitis B virus, Epstein-Barr virus, human T-cell leukaemia virus and human immunodeficiency virus (HIV) (Bower and Waxman, 2006).

Correa, (2003) reported that Helicobacter pylori, a type of Gram-negative bacteria contribute to chronic gastritis, peptic ulcer, and low grade gastric mucosa associated lymphoid tissue (MALT) lymphoma and the pathogenesis of gastric cancer. Other
bacteria like *Salmonella typhi* is involved in the development of gall bladder cancer (Lazcano-Ponce *et al.*, 2001), *Streptococcus bovis* promotes colon neoplasia (Ellmerich *et al.*, 2000) while *Chlamydia pneumoniae* increases the risk of lung carcinoma (Littman *et al.*, 2004).

The role of parasites in causing cancers is not well researched and the evidence of parasite infections in contributing to various cancers is low. However, Mostafa *et al.*, (1999) claimed that *Schistosoma haematobium* is linked to bladder cancer and its infestation in developing bladder cancer was attributed to the inactivation of p53 and over-expression of Bcl-2. In another unrelated study, the infection of the *Opisthorchis viverrini* liver fluke increased the risk of cholangiocarcinoma, which is accounted for approximately 4% of all human malignancies (Parkin *et al.*, 2001).

### 2.3 Cancer Chemoprevention

Cancer chemoprevention was defined as a strategy used to reverse or suppress carcinogenesis through the administration of natural or synthetic compounds at a pre-malignant cancer stage (Kelloff, 2000). In other words, chemoprevention is an approach to inhibit the invasion of cancer through usage of natural or pharmacological agents (Flora *et al.*, 2001).

Sufficient epidemiological studies have revealed that there is a possibility of preventing cancer and other chronic illness and it is believed that the intake of suitable protective dietary factors and/or pharmacological agents plays an important role in the cancer prevention strategy (Ferguson *et al.*, 2005). These agents must meet certain requisites as it is eventually being administered by humans. It must be affordable and easily available, produced, stored and administered. A
chemopreventive agent must also prove high efficacy and most crucial, is not hazardous to health (Flora and Ferguson, 2005).

In addition to that, it is reminded that it is uncommon for a chemopreventive agent to be specialized in a particular level of intervention study. These agents are known to be involved in multiple prevention mechanisms as triggered by their various properties (Flora et al., 2001). Hence, a chemopreventive agent may play many different roles at different levels of intervention.

Cancer chemoprevention can be applied at three different levels, depending on the target of intervention. Firstly, it is introduced as primary prevention for ‘prevention of occurrence of disease’; secondary prevention as ‘early detection and intervention, preferably before the condition is clinically apparent, and has the aim of reversing, halting or at least retarding the progress of a condition’ and tertiary prevention to ‘minimize the effect of the disease by preventing complications and pre-mature deteriorations’ (Last, 1986).

In other words, primary prevention is subjected to healthy individuals with the purpose of inhibiting carcinogenesis while secondary prevention is introduced to individuals already affected by a benign tumour in the hopes of regressing tumour progression. The objective of tertiary prevention is to prevent cancer recurrence, invasion and metastasis (Flora et al., 2001).

**Primary Prevention**

The target of primary prevention is to keep none or minimal contact with the risk factors that may trigger carcinogenesis. According to Flora and Ferguson, (2005), by
increasing the host organism resistance against the attack of mutagens and carcinogens, either extracellular or non-target cells, the occurrence of cancer can be deterred. The few primary prevention strategies include the inhibition of mutagens and/or carcinogens formation and uptake and inhibition of mutation and cancer initiation in targeted cells by cellular mechanisms such as modulation of DNA metabolism and repair and inhibition of oncogene expression. Anti-proliferation and apoptotic activities are strategies involved in interrupting tumour promotion (Flora et al., 2001). It was suggested by Loeb et al., (2003) that by inhibiting mutation, the onset of cancer can be delayed.

As such, the consumption of dietary anti-cancer nutrients may be suggested as the important source of chemoprevention. For instance, it is evident that the consumption of Allium and Brassica vegetables such as garlic, onion, broccoli and cauliflower greatly benefits in reducing the incidences of various cancers. The protective effects of these plants were attributed by their bioactive properties, namely diallyl sulfides and isothiocyanates. These bioactive compounds induce the detoxification pathways which is the human body’s first line of defence to help remove carcinogens and mutagens by stimulating the glutathione-S-transferase enzyme (Chen et al., 2004; Hecht, 1999).

Secondary Prevention

In secondary prevention, a variety of mechanisms are exploited to achieve the goal of inhibiting the progression of carcinogenesis. The progression of carcinogenesis is inhibited through a few mechanisms which include the inhibition of genotoxic effects and protease enzymes, modulation of signal transduction and the hampering of angiogenesis. Hormone therapy is also applied to prevent the progression of
selected cancers such as breast, ovary and prostate cancer. Antioxidant activity and free radical scavenging also play a vital role in diminishing carcinogenesis progression (Flora and Ferguson, 2005).

An example of secondary intervention is in the prevention of skin carcinogenesis. Due to the multi-step of skin carcinogenesis, it was suggested to better inhibit promotion than treating the resultant cancer (Sporn and Suh, 2000). Hence, according to Nomura et al., (2000), theaflavins from black tea inhibits the activation of UVB-induced AP-1 promoter gene thus interrupting the further promotion of skin carcinogenesis.

Tertiary Prevention
In tertiary prevention, the main target of intervention is on invasion and metastasis. The goal of intervention is to inhibit invasion and metastasis of cancer. This stage of chemoprevention involves the inhibition of protease enzymes that are involved in basal membrane degradation and extracellular matrix interaction. By affecting the cell adhesion properties, tumour invasion is interrupted. Cell differentiation and proliferation are also hampered.

Boissier et al., (2000) reported that bisphosphonates prevents the attachment of invasive cancer cells from the breast or prostate to the extracellular matrix and the vascular beds of the host cell hence hindering bone metastasis. This bioactive compound also exerts anti-angiogenesis activity against breast cancer cells, preventing the formation of new capillaries (Coleman, 2001).
2.3.1 Mechanisms of Cancer Chemoprevention

This diverse carcinogenicity can be counteracted by attacking multiple key pathways simultaneously in a limited time frame so that the situation does not deteriorate before it is even treated. Thus it is important to know the originality of the cancer so that the suitable chemopreventive mechanism can be applied. According to Steele and Kelloff, (2005), there are two diverse types of chemoprevention mechanisms that can be applied to cancer therapy: anti-mutagenic and anti-proliferative.

Anti-mutagens

Anti-mutagens are agents that are responsible for inhibiting the activation of carcinogens and carcinogen uptake in the body. Anti-mutagens also detoxify the activated carcinogens and play a role in increasing the fidelity of DNA repair. According to Arora and Shukla, (2003), indole-3-carbinol (I3C) help modulate the expression of the multidrug resistance-1 (MDR-1) gene transcript that was induced by vinca-alkaloids as over expression of MDR-1 will enhanced the resistance of cancer against chemotherapy (Bower and Waxman, 2006). In 2005, Arora et al., had demonstrated that I3C was a non-toxic, specific and potential MDR modulator in the K562 human leukaemic cells. Other agents that were found to exhibit similar effect were diallylsulfides found in Allium families and polyphenols found in tea such as theanine and (-)-epigallocatechin gallate (Sadzuka et al., 2000).

Anti-proliferatives

On the other hand, anti-proliferatives inhibit angiogenesis, DNA synthesis, oncogene activity and also polyamine synthesis. Both apoptosis and terminal differentiation are induced by the presence of anti-proliferatives such as retinoid and vitamin D while folic acid and budesonide balances the methylation of DNA (Steele
and Kelloff, 2005). Sgambato et al., (2001) had shown that resveratrol, a broadly distributed phenolic compound had great anti-proliferative activity whereby this compound inhibits both normal and cancerous cells proliferation by exhibiting convincing IC$_{50}$ values in the range of 20.0 and 100.0 µM. Retinoid was reported to induce differentiation in epithelial cells retarding the progression of carcinogenesis (Sun and Lotan, 2002).

**Anti-inflammatory Agents**

The involvement of anti-inflammatory agents is important to bring down the proliferation rate of cancerous cells. According to Steele and Kelloff, (2005), the inflammatory mechanism is induced by the arachidonic acid metabolism pathway where inflammatory molecules such as prostaglandin, thromboxane, leukotrienes and hydroxyeicosatetraenoic acids are produced. These molecules intensively stimulate and enhance the evolution of the human cancers at the cellular stage. For example, aspirin (acetylsalicylic acid) inhibits the cyclooxygenase-2 (COX-2) enzyme resulting in the decrease production of prostaglandin E2 (PGE2) that triggers inflammation and also facilitates the development of cancer (Kleinsmith, 2006).

**Oncogene Inhibitors**

Another cancer chemopreventive mechanism is through oncogene inhibitors. Oxford and Theodorescu, (2003) demonstrated that members of the ras monomeric G oncoproteins were involved in multiple cellular processes and often found over expressed in tumours. The most common mutations found are K-ras mutations in cancers of the pancreas, lung and colon, while H-ras mutations are found in bladder and kidney cancers (Adjei, 2001; Vageli et al., 1996). Limtrakul et al., (2001) had
demonstrated that the intake of curcumin can reduce the c-Ha-ras and c-fos proto-oncogenes expression after topical application of a carcinogen in mice.

**Anti-angiogenesis**

When tumourigenesis is initiated and the tumour eventually takes its place at a distant site, it is important that now, the tumour is able to develop its own vascular bed in order to grow and maintain the flow of nutrients from the bloodstream. The application of a natural or synthetic agent to disrupt the angiogenesis is crucial to ensure that progression of tumourigenesis is retarded (Steele and Kelloff, 2005).

Kimura and Okuda, (2001) had shown that intraperitoneal administration of resveratrol was shown to inhibit tumour growth, metastasis and angiogenesis of implanted cancerous lung cells in mice. Retinoids, non-steroidal anti-inflammatory drugs (NSAID) and selective estrogen receptor modulators (SERM) like tamoxifen and steroid aromatase inhibitors were also reported to have anti-angiogenesis properties (Sharma et al., 2001).

**Apoptosis**

Apoptosis, or programmed cell death can be induced to inhibit the progression of carcinogenesis. This mechanism was reported by Shi and Gould, (2002) in mammary carcinoma cells that cytostasis and apoptosis were induced in the presence of a monoterpenoid anti-cancer agent, perillyl alcohol. Histone deacetylase (HDAC) inhibitors such as trichostatin A (TSA) and sodium butyrate (NaB) can also induce apoptosis (Marks et al., 2000) and differentiation of transformed cells (Zhou et al., 2000).
A synthetic HDAC, 4-dimethylamino-N-(6-hydroxycarbamoyl-hexyl)-benzamide (M344) was demonstrated to induce growth inhibition, cell cycle arrest and apoptosis in human endometrial and ovarian cancer cell lines by Takai *et al.*, (2006) while a synthetic anti-tumour agent was reported to inhibit tumourigenesis *in vitro* and *in vivo* through the induction of programmed cell death via the mitochondria-mediated caspase activation pathway in the Lewis lung carcinoma (LLC) animal model (Lee *et al.*, 2006).

**Dietary Antioxidants**

A novel way of preventing the occurrence of oxidative deterioration is through the simple consumption of vegetables and fruits which have been proved to provide protection against various diseases. The natural occurring polyphenols such as epigallocatechin-3-gallate in green tea were found to inhibit oxidative damage of DNA (Wei *et al.*, 2006) and regulation of cell growth and differentiation (Park and Surh, 2004). Vitamin C at non-toxic levels was used alongside chemotherapy to increase the cytotoxicity of cisplatin and etoposide against HeLa cells, a human cervical cancer-derived cell line, by stabilizing the p53 protein (Reddy *et al.*, 2001). Other naturally occurring antioxidants include quercetin, β-carotene, lycopene, curcumin and catechins.

**2.4 Natural Product in Cancer Chemoprevention**

In recent years, there has been booming interest in natural product research, where scientists from all over the world are exploiting the therapeutic values of natural products, especially those derived from plants. Natural products or its derived drug had dominated approximately one quarter of the best-selling drug market worldwide in both 2001 and 2002 (Butler, 2004). It was reviewed by Rates, (2001) that the
National Cancer Institute (NCI) had tested 33,000 samples for anti-tumour activity and more than 50,000 plant samples for anti-HIV activity. As natural products were declared to be safe and non-toxic to human, they were ideal for long term application and effective as a chemoprevention drug (August, 2003).

Generally, diet that is rich in vegetables, fruits and fibres is an important protective measure as it is associated with a reduced risk of cancer (Reddy et al., 2003). Many natural compounds from vegetables and herbs exert chemopreventive properties against carcinogenesis. Kulscar, (2000) had indicated that 13 compounds found in the blood stream actually inhibit the cancerous cell growth in vitro and in animals, synergistically in cancer therapy. Between 1940 and 2002, 40% of the anti-cancer drugs were natural products per se or natural product derivatives while the other 8% were natural product mimics (Newman et al., 2003). It was believed that these plant-derived constituents are responsible in playing an important protective role against many diseases including cancers to its consumer.

**Tea**

The tea polyphenol constituents have shown potential inhibitory activity against human carcinogenesis of different organ sites. Green tea intake was reported to reduce breast cancer risk (Sun et al., 2006) and has greater chemopreventive potential than black tea in preventing prostate cancer (Chhabra and Yang, 2001). The protective effect of tea against lung cancer was also observed (Bonner et al., 2005). In Japan, green tea consumption was associated with a lowered risk for gastric cancer (Tsubono et al., 2001).
Fruits
A carbohydrate constituent in the rind of *Punica granatum* or better known as pomegranate had demonstrated free radical scavenging activities (Rout and Banerjee, 2007) and help attenuate the UV-induced oxidative stress and stress-induced molecular pathways related with a high risk of skin carcinogenesis (Afaq *et al*., 2007). Resveratrol is a polyphenol found abundantly in the skin of red grapes was reported to interrupt the process of carcinogenesis and suppress angiogenesis and also metastasis (Baliga *et al*., 2005).

Stoner *et al*., (2007) researched that consumption of berries can help reduce levels of carcinogen-induced DNA damage and also inhibit the promotion and progression of carcinogenesis in mice. Persin and two of its analogues found in avocado leaves showed inhibitory effects against the formation of reactive oxygen species and nitric oxide thus suggesting their potential as cancer chemopreventive agents (Domergue *et al*., 2000).

Vegetables
β-glucans, a polysaccharide be found in fungus inhibits the activation of carcinogenesis by carcinogens such as benzo[a]pyrene (Hashimoto *et al*., 2002). Clitocine, a bioactive compound in mushroom *Leucopaxillus giganteus* had exerted significant anti-proliferative properties against HeLa cells in a dose- and time-dependent manner (Ren *et al*., 2008). Quercetin, a common dietary flavonoid, is found mostly in onions, hinders oxidative damage to the DNA which subsequently prevents carcinogenesis (Yang *et al*., 2004) and protects and inactivates harmful effects of chelate metal ions (Kaiserova *et al*., 2007). Lee *et al*., (2008) had demonstrated that [6]-gingerol from ginger can inhibit metastasis *in vitro* by
preventing cell adhesion, invasion, motility and extracellular matrix degrading enzymes activity.

**Vitamins**

Gasowska-Giszczak *et al.*, (2005) suggested that vitamin A and its analogues may be used to prevent the progression of cervical lesions at the early stage while vitamin D has been related to the prevention of colorectal cancer in human at optimal dose of 1000-2000 IU per day (Gorham *et al.*, 2007). Bermudez *et al.*, (2007) found that vitamin E had significantly suppressed the activity of endogenous telomerase, a ribonucleoprotein in human ovarian cancer cell lines following a dose- and time-dependent manner.

**Other Supplements**

Micronutrients and trace elements such as selenium, iron, potassium, calcium, riboflavin, folic acid and coenzyme-Q (ubiquinone) are potent natural antioxidants. These nutrients can be found in cereals, wheat, nuts and most vegetable food. According to Wang *et al.*, (2001), selenium had demonstrated apoptotic properties while in Lu and Jiang, (2001); selenium was evident in reducing intra-tumoural microvessel density and inhibit the expression of vascular endothelial growth factor in induced-mammary carcinogenesis. By inhibiting angiogenesis, invasion and metastasis can be hindered.

Coenzyme-Q is synthesized in all tissues and cells and this lipid is found in all membranes (Turunen *et al.*, 2004). The physiological function of this compound lies in the inner membrane of the mitochondria. Its main functions include electron carrier, generator of superoxide anion radical through auto-oxidation of
ubisemiquinone and free radical quencher (James et al., 2004; Turunen et al., 2004). Coenzyme-Q was claimed to regenerate vitamin E from its radical (Wang et al., 2004a), enhances the cytotoxic effects of the natural killer (NK) cells (Ravaglia et al., 2000).

The intake of other supplements such as calcium may enhance the function of vitamin D working synergistically to reduce cancer risk (Lappe et al., 2007; Grau et al., 2003). Folate was associated with the reduction of colorectal cancer risk (Sanjoaquin et al., 2005) but high folate profile may be disadvantageous and may as well intensify the risk of cancer and DNA damage (Kim, 2004).

2.5 Anti-cancer Agents

Anti-cancer activity can be exerted through a wide variety of mechanisms mostly targeting DNA, directly or indirectly by anti-cancer agents. Anti-cancer agents are drugs used to control the growth of cancerous cells and/or treat malignancies by eradicating cancer cells without harming the normal cells (Chabner and Thompson, 2003). An important characteristic of an anti-cancer drug is to be able to induce cancer cell apoptosis and as such differentiates between an anti-cancer drug and a toxic compound (Frankfurt and Krishnan, 2003). The classification and mechanism of action of these anti-cancer agents are tabled in Table 2.1.
<table>
<thead>
<tr>
<th>Class of Agent(s)</th>
<th>Examples</th>
<th>Drug Mechanism(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytotoxic Agents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkylating and platinating agents</td>
<td>Cyclophosphamide Chlorambucil Mephalan Cisplatin</td>
<td>Cross-linking with DNA causing breaks in DNA and error in DNA transcription</td>
</tr>
<tr>
<td>Anti-metabolites</td>
<td>Methotrexate 6-Mercaptopurine 5-Fluorouracil Cytarabina</td>
<td>Folic acid antagonist Inhibits the production of purine metabolites Inhibits the production of pyrimidine metabolites</td>
</tr>
<tr>
<td>Topoisomerase inhibitors</td>
<td>Irinotecan Topotecan Doxorubicin Daunorubicin</td>
<td>Inhibits topoisomerase I enzyme Inhibits topoisomerase II enzyme Blocks the topoisomerase II enzyme and prevents DNA synthesis and repair</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Bleomycin Mitomycin Doxorubicin Daunorubicin</td>
<td>Promoting DNA breaks DNA cross-linking agent Inhibits topoisomerase II enzyme Inhibits topoisomerase II enzyme</td>
</tr>
<tr>
<td>Anti-microtubules</td>
<td>Vincristine Vinblastine Vinorelbine Taxol</td>
<td>Interrupts the division of cancer cells by preventing the formation of spindle at metaphase Induce polymerization of tubulin and arrest cells at metaphase</td>
</tr>
<tr>
<td><strong>Hormone Therapies</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective estrogen receptors modulators (SERMs)</td>
<td>Tamoxifen</td>
<td>Estrogen receptors inhibitor (in breast)</td>
</tr>
<tr>
<td>Aromatase inhibitors</td>
<td>Arimidex</td>
<td>Inhibits the production of extragonadal estrogen</td>
</tr>
</tbody>
</table>
Table 2.1 continued

<table>
<thead>
<tr>
<th>Category</th>
<th>Drug</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-androgens</td>
<td>Bicalutamide</td>
<td>Androgen receptors inhibitor (in prostate)</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td>Prednisone</td>
<td>Glucocorticoid inhibitor</td>
</tr>
<tr>
<td><strong>Immunomodulatory Agents</strong></td>
<td>Interferon</td>
<td>Retards cancer cells' division and strengthens the body's immune system</td>
</tr>
<tr>
<td></td>
<td>Interleukin-2</td>
<td>Stimulates body's defence mechanism</td>
</tr>
<tr>
<td><strong>‘Novel’ Agents</strong></td>
<td>Imatinib</td>
<td>Interrupts growth stimulatory pathways</td>
</tr>
<tr>
<td>Signalling inhibitors</td>
<td>Erlotinib</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gefitinib</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bortezomib</td>
<td></td>
</tr>
<tr>
<td>Anti-angiogenesis</td>
<td>Bevacizumab</td>
<td>Inhibits the formation of new blood vessels by binding to vascular endothelial growth factor (VEGF)</td>
</tr>
<tr>
<td></td>
<td>Thalidomide</td>
<td>Inhibits angiogenesis and tumour necrosis factor</td>
</tr>
</tbody>
</table>

(Adapted from Bower and Waxman (2006); Kleinsmith (2006); Watson et al. (2006); Chabner and Thompson (2003))

### 2.5.1 Cytotoxic Activity

It is important to treat cancer and by treating cancer, it simply means to kill the cancer cells, and cytotoxic agents are compounds used to kill the cancer cells (King, 2000). As for the term ‘cytotoxic activity’, it is defined as the attempt to arrest the proliferation of cancerous cells by influencing apoptosis using plants’ natural ability (Dewick, 1996). In other words, cytotoxic activity can be described as the activity to kill cancerous cells by using a cytotoxic agent.
The assumption that the regulation of apoptosis in plants is mediated by its very own chemical content hence; it is most likely that plants can provide us with an important source of agents that are involved in the modulation of apoptosis. This correlates with the findings from different studies that showed that numerous food items and herbal medicines exerted their toxicity against cancerous cells by inducing apoptosis (Thatte et al., 2000).

2.5.2 Cytotoxicity Screening

Toxicity and general screening of compounds were predicted in human using in vitro cytotoxicity assays (Scheers et al., 2001). The suitable in vitro cell system must be chosen for the right purpose of cytotoxicity testing. In the early stage of discovery, it is important to include cytotoxicity screening to help identify potential cytotoxic agents. Different cytotoxicity assays will reveal different results depending on the cytotoxicity assay employed and the test agent used (Weyermann et al., 2005). Thus, it was suggested that more than one cytotoxicity assay should be employed in studies evaluating the viability of cells to increase the reliability of the results obtained (Fotakis and Timbrell, 2006).

The cytotoxicity tests that are practical, sensitive, easily reproduced and predictive are always favoured (Fotakis and Timbrell, 2006). The few preferred cytotoxicity assays that are frequently used in numerous studies are NR assay (Berckmans et al., 2007), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) salt assay (Mesia et al., 2008) and lactate dehydrogenase assay (LDH) (Fotakis and Timbrell, 2006). Other cytotoxicity assays are uridine uptake inhibition (Valentin-Severin et al., 2002), the protein assay and the AlamarBlue assay (Holst and Oredsson, 2005).
Briefly, the NR assay is used to measure damages in the cell membrane by monitoring the amount of dye penetration into the lysosomes of viable cells (Scott-Fordsmand and Weeks, 2000). The MTT tetrazolium salt assay determines the cytotoxic effect of a test agent against uninjured cells which is reflected by the reduction of formation of blue formazan from the yellow MTT tetrazolium salt by the succinate dehydrogenase in the mitochondria of the cell (Tan et al., 2008). The LDH assay measures cell cytolysis based on the levels of lactate dehydrogenase released in the culture supernatant (Bellocci et al., 2008).

The protein assay is an indirect method of measuring the viability of cells through the presence of their protein content (Fotakis and Timbrell, 2006). As for the AlamarBlue assay, the presence of pink fluorescence detected in the system tested reflects the metabolic activity of the cells where the more intense the pink fluorescence, the higher the viability of the cells (Holst and Oredsson, 2005). Among these cytotoxicity assays, the NR assay is one of the most sensitive cytotoxicity assays in detecting early toxicity by revealing statistically significant results between the controls and the treated cells (Fotakis and Timbrell, 2006).

The NR Assay

NR is a supravital dye and it is used to estimate the viability of cells. In other words, NR indicates the acute toxicity of known xenobiotics or conditions which is reflected by NR uptake by the cells and also the integrity of intact cell membrane and only the viable, uninjured cells accumulate NR in their lysosomes. In other words, the uptake of the NR dye is proportional to the lysosomal content of the cell (Scott-Fordsmand and Weeks, 2000).
An increase in NR uptake by the cells could mask a cytotoxic effect where the amount of dye released reflects cell death and it is determined using the spectrophotometry method where the optimum sensitivity of NR is obtained at 540 nm (Verhulst et al., 1998). The higher concentration of a known compound treated unto the cells, the higher the cell death rate (George et al., 2000).

The NR assay is more accurate than any other toxicity assay because the uptake of NR is not biased by occasional microbial contamination, which can lead to an overestimation of cell viability. In this assay, the morphology of the cell is well preserved thus allowing the cell viability to be evaluated qualitatively using photomicrographs of the cell layers both before and after dye elution (Ciapetti et al., 1996).

Other advantages of the NR assay include easy handling, many replicates, economic as the reagents are easily obtained and the assay only involves the usage of common laboratory equipments. However, the most important fact is that the NR assay can be performed within a short assay time (approximately three hours or less) and still retaining its high sensitivity level in estimating the cell viability and/or growth (Ciapetti et al., 1996).

The cytotoxic activity was expressed as the IC$_{50}$ value estimated from the graphical interpolation of the dose-response curve, which is defined as the concentration of extract that causes 50% of inhibition or cell death (Chiang et al., 2003; Chen et al., 1988; Geran et al., 1972). The extrapolated IC$_{50}$ value from the dose-response curve is more consistent when compared to value-based (Chapuis et al., 1988). The extract that gave an IC$_{50}$ value 20.0 µg/ml or less is considered as active. However, plant
extracts exhibiting IC$_{50}$ value of more than 30.0 µg/ml were considered not active and does not require further testing (Chen et al., 1988; Geran et al., 1972).

2.6 Free Radicals

Free radicals are any atom or molecule containing an unpaired electron. The free radicals are reactive species because of the presence of the unpaired electron. It is highly unstable and aggressively seeks for an electron to stabilize itself again by ‘stealing’ an electron from the nearest molecule, leaving that particular molecule with insufficient electrons. Now, this molecule will seek to ‘steal’ its own electron from another molecule and so on in a chain reaction. Each time these free radicals happen to take up an electron from a normal cell, the molecule structure will be interrupted and the cell might not be able to function and/or die (Kleinsmith, 2006).

The damage done by these free radicals is known as oxidation and these chain reactions, if not stopped, will create more damages to the body in a long term. When the oxidant particles trespass into a cell seeking for electrons, it breaks the cell membrane and disrupts the cell’s normal function. The cell defense mechanism is brought down when the free radicals hit the lysosomes (Kleinsmith, 2006).

Consequently the lysosomes burst, releasing the self-destructive enzymes, which will then cause massive damages inside the cell. The DNA will also be damaged by these free radicals and this in turn can lead to cancer and other deadly diseases (Miller, 2005). These molecules were also believed to exacerbate cell injury that eventually leads to aging (Gulcin et al., 2002).
2.6.1 Free Radical Source

Free radicals can be found endogenously and exogenously. The endogenous free radicals are formed inside the human body as by-products of normal functions while the presence of external stimuli enhances the production of exogenous free radicals.

**Endogenous**

Inside the human body, free radicals are generated during aerobic processes. Mitochondrial oxidative phosphorylation, cytochrome P450 metabolism, peroxisomes and activation of inflammatory cells contribute to the formation of free radicals. Oxygen is broken down during redox reactions to produce energy via a series of sequential electron reductions that consequently forming superoxide radicals, hydrogen peroxides and hydroxyl radicals (Pappa et al., 2007).

These radicals are important as an intermediate in the body’s defence against invading microorganisms. However, excessive free radicals could pose danger to the cells for they may kill the cells and subsequently causes damage to tissues (Vimala et al., 2003).

**Exogenous**

The production of exogenous free radicals is enhanced by the high consumption of solid fats, grilled or roasted food, processed food products and stale food. Environmental and occupational factors such as lifestyle, exposure to radiation, pollution and pesticides also contribute to generation of free radicals (Martin-Moreno et al., 2008; Vimala et al., 2003)
However, when free radicals are produced sufficiently at the right time and place, they play an entirely different role in promoting if not, maintaining one’s health. For instance, our white blood cells produce free radicals to savage invading microorganisms (Inserra et al., 1997) while super oxides regulate cell growth and intercellular signalling processes (Papas, 1998).

2.6.2 Types of Free Radicals

Generally, most of the occurring free radicals are oxygen-based molecules (Miller, 2005) but there are other types of radicals that exist depending on the major atom(s) of the species, for instance, the nitrogen-centred species and sulfur-centred species.

Reactive Oxygen Species (ROS)

The oxygen-centred species are known as ROS. Endogenous ROS are often by-products of oxidative phosphorylation or exposure of ionizing radiation, from phagocytic cells and lipid peroxidation (King, 2000). The few important reactive oxygen species are superoxide radicals (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), singlet oxygen (¹O$_2$), peroxyl (RO$_2^°$) and hydroxyl radicals (OH˚) (Miller, 2005).

These radicals induce permanent changes in the DNA sequence in the form of point deletions, mutations, the amplification and rearrangement of the genes subsequently activating the proto-oncogenes or inactivating the tumour suppressor genes (reviewed in Goetz and Luch, 2008) and have been implicated to more than 100 disorders (Buyukokuroglu et al., 2001). The production and destructive cycle of ROS is illustrated in Figure 2.1.
Figure 2.1 The formation and depletion of ROS (Adapted from King, 2000)

i) Superoxide, $O_2^-$ and Hydrogen Peroxide, $H_2O_2$

The $O_2^-$ radical is formed in the microsomal and mitochondrial membranes through enzymatically catalyzed one-electron reduction of triplet oxygen (Halliwell and Gutteridge, 2007) and can be converted into $H_2O_2$ by super oxide dismutase (SOD) or other non-enzymic dismutation. On the other hand, cellular $H_2O_2$ is produced via autoxidation of reactive chemicals during redox cycling between microsomal and mitochondrial electron transport sites, peroxisome metabolism and SOD activity (Chance et al., 1979).

$H_2O_2$ is a precursor and not a free radical. Nevertheless, $H_2O_2$ on its own is directly oxidative against other proteins such as the transcription factor, nuclear factor-$\kappa$B (NF-$\kappa$B) (King, 2000). Both $O_2^-$ and $H_2O_2$ are likely to target at intracellular substances which they can do direct damage but nevertheless, their reactivity is limited at their site of formation. $H_2O_2$ passes all the biological membrane easily as
compared to $O_2^-$ hence; spreading its reactivity and possible toxicity (reviewed in Boots et al., 2008).

ii) Hydroxyl ($OH^\circ$) and Peroxyl ($RO_2^\circ$) Radical

$OH^\circ$ is abundantly found in cells and it plays an important role in DNA damage by attacking the nucleotide bases resulting in the breaking of DNA strand (Schyman et al., 2008). It is the most reactive ROS and thus the most damaging because it will react with any molecule it encounters (Galli et al., 2005). The main *in vivo* source of $OH^\circ$ is from the Haber-Weiss reaction (Kehrer, 2000).

$$O_2^\circ + H_2O_2 \rightarrow O_2 + OH^\circ + OH \text{ (Haber-Weiss reaction)}$$

As for $RO_2^\circ$ radicals, they are formed when carbon-centred radicals produced react with oxygen and these radicals are the key intermediate in oxidation of organic compounds (Alfassi, 1997). The formation of peroxyl radicals is the major chain-propagating step in auto-oxidation of lipoproteins and biological membranes components (Chilsom and Steinberg, 2000).

iii) Singlet Oxygen ($^1O_2$)

Singlet oxygen is not classified as a radical as it does not have an unpaired electron. The transfer of energy from excited-state endogenous or exogenous sensitizers to ground-state molecular oxygen can produce $^1O_2$ (Cadet et al., 2006). Otherwise, this ROS can be formed as a product of peroxidase enzymes activities (Davies, 2004). $^1O_2$ mainly target proteins as proteins have the closest proximity to the lipids in the cell membrane which was proposed as the primary generation site for singlet oxygen (Davies, 2003).
Reactive Nitrogen Species (RNS)

Nitrogen-centred species are now considered as one of the major components of oxidative burst. Varieties of nitrogen oxides have been established and nitric oxide (NO°) is the most researched form of nitrogen oxides. Other nitrogen oxides are nitrite (NO\textsubscript{2}°), nitrogen peroxide (NOO°) and peroxynitrite (ONOO°) and (Lopez et al., 2007). NO° is produced in the cells by nitric oxide synthases (Drew and Leeuwenburgh, 2002) and it is not as reactive as NOO° and ONOO°. NO°, NOO° and ONOO° can be formed endogenously however; these radicals can also be produced as carcinogenic by-products of nitrogen oxide (NO\textsubscript{2}) of tobacco smoke (King, 2000).

Reactive Sulfur Species (RSS)

As for sulfur-centred species, these radicals are formed when thiols are altered in cellular oxidative defence systems in the human body (Ashby and Aneetha, 2004). These reactive sulfur species include thiyl (RS°), polysulfide (RS\textsubscript{n}), sulfinyl (RS\textsuperscript{O}°), sulfonyl (RSO\textsubscript{2}°) radicals and other anion and cation radicals. In human, the lactoperoxidase system produces unstable intermediate oxidation products of thiocyanate (SCN\textsuperscript{−}) such as hypothiocyanite (OSCN\textsuperscript{−}) which possess anti-bacterial activity (Furtmueller et al., 2002).

2.7 Oxidative Stress

One of the most important mechanisms contributing to cancer is oxidative stress. Oxidative stress is defined as ‘a cell’s state characterized by excessive production of ROS and/or a reduction in antioxidant defences responsible for their metabolism’ (Franco et al., 2008). When the equilibrium of antioxidant defence system and the
formation of ROS are interfered, the oxidative stress subsequently causes irreversible oxidative damages to the DNA (Baublis et al., 2000).

It has been proposed that direct oxidative damages of the DNA by ROS will lead to ROS-induced carcinogenesis and hydroxyl radicals generated through iron-mediated processes via the Fenton reaction are mainly associated with these damages. It is such that the hydroxyl radicals will produce more mutagenic products such as purine, pyrimidine, peroxynitrite and hypochlorous acid which may be relevant to carcinogenesis (Cooke et al., 2003; Hawkins and Davies, 2002).

Inflammation is another major contributor of oxidative stress. The response of inflammatory cells towards stimuli subsequently produces ROS and nitric oxide. ROS may initiate the production of various cytokines via activation of transcription factor, NF-κB and incorrect regulation of this factor results in cancer (Rahman, 2002). Bruce et al., (2000) reported that the presence of nitric oxide in the system allows the formation of peroxynitrite, a more reactive nitrogen species, directly interacting with DNA or other macromolecules such as lipids, initiating free radical chain reactions. Uncontrolled chain reactions may cause severe oxidative damages which result in carcinogenesis.

Simply put the imbalance between the production of and protection against ROS results in oxidative stress (Vimala, 2008). Hence, this is where the application of antioxidants plays an important role in preventing oxidative damages caused by oxidative stress and subsequently protects against carcinogenesis. Figure 2.2 simplifies the inter-relation of ROS, oxidative stress and antioxidant.
2.8 Antioxidant

Antioxidant is defined as ‘any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate’ (Halliwell, 1995). In other words, antioxidants can be described as substances that slow the rate of oxidation reactions and these substances inhibit the process by removing free radicals which can lead to cell and tissue destructions (Martin and Hine, 2000).

2.8.1 Types of Antioxidants

Antioxidants can be classified as endogenous and exogenous antioxidant based on their sources. Endogenous antioxidants are naturally produced by the human body and are considered as enzymatic antioxidants. These antioxidants are catalyst and are efficiently recycled. A small amount of these endogenous antioxidants is sufficient for protection (Diplock et al., 1998). SOD, CAT and GSH.Px are categorized as endogenous antioxidants.
Exogenous antioxidants are primarily plant-derived and these substances can act as reducing agent, free radical scavengers and/or quenchers and metal chelators (Mathew and Abraham, 2006). Vitamins, carotenoids, phenolic compounds and catechins are examples of exogenous antioxidants that are obtained through food diet.

SOD
The reduction of a single-electron superoxide into hydrogen peroxide and oxygen is catalyzed by SOD. In mammalian cells, SOD exists in the form of Cu/Zn-SOD in cytosol and mitochondria while Mn-SOD can be found only in the matrix of mitochondria (Serafini, 2006).

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

CAT
CAT is responsible for the two-electron reduction of hydrogen peroxide into water and oxygen. This enzyme is found in the cytoplasm of erythrocytes and in peroxisomes of other cells (Serafini, 2006).

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]

GSH.Px
GSH.Px is an important enzyme which is involved in the elimination of hydrogen peroxide. GSH.Px catalyst the conversion of lipid hydroperoxides (LOOH) into their corresponding alcohols (LOH) and as such, the enzyme itself is oxidized into GSSG.
GSH.Px is renewed to the antioxidant network in the human body when GSSG is reduced by NADPH-dependent glutathione reductase (Serafini, 2006).

\[
\text{LOOH} + 2\text{GSH.Px} \rightarrow \text{LOH} + \text{GSSG} + \text{H}_2\text{O} \quad (\text{Conversion of LOOH})
\]

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow \text{NADP}^+ + 2\text{GSH.Px} \quad (\text{Reduction of GSSG})
\]

**Vitamins**

Vitamins are essential to the body because the human body cannot produce its own vitamin source and solely depends on food and supplements for its source. Vitamins such as vitamin C and E are crucial antioxidants that help fight oxidative stress and also prevent cancer. Vitamin C acts as a cytotoxic agent to several cancer cell lines and also is an efficient free radical scavenger (Reddy *et al.*, 2003; Clement *et al.*, 2001; Reddy *et al.*, 2001; Davey *et al.*, 2000). Alternatively, vitamin E is a lipid-phase free radical scavenger (Davey *et al.*, 2000). The combination usage of vitamin C, E and polyphenol compounds may further enhance the biological systems in our body (Liao and Yin, 2000).

**Carotenoids**

Carotenoids are yellow, orange and red plant pigments found in fruits and vegetables. β-carotene, lutein, cryptoxanthine and lycopene are prominent members of this phytochemical group. β-carotene, γ-carotene and β-cryptoxanthine are lipid-soluble precursors of vitamin A and the presence of conjugated double bonds in their structure (Johnson, 2002) made them potent antioxidants that help prevent cancer and other chronic diseases (Bowen *et al.*, 2002; Aggarwal and Rao, 2000).
Phenolic Compounds

The potential of phenolic compounds as food antioxidants is attributable to the number of present hydroxyl groups and where it is situated in their structure. The stability of these compounds in a system also favours their potential as antioxidants (reviewed in Podsdek, 2007; Bandoniene and Murkovic, 2002). Flavonoids, curcuminoids, stilbenes and anthocyanidins are examples of plant-derived phenolic compounds. Phenolic compounds were claimed to be ROS scavengers (Abdulla and Gruber, 2000) and inhibits carcinogenesis via various mechanisms (Bednar et al., 2007; Gerhauser et al., 2003; Ahmad et al., 2001; Nakagawa et al., 2000).

Catechins

Catechins, is commonly found in Camellia sinensis plant, the common source of green tea, black tea and oolong tea. Catechins are potent metal ion chelators, reactive species trappers (Hou et al., 2005) and also good donors for hydrogen-bonding (Fang et al., 2003). The prominent catechin compounds consist of (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC), with EGCG being the most abundant and active catechin (Kuzuhara et al., 2008).

2.8.2 Mechanisms of Antioxidant Defence

The mechanism of antioxidant defence can be categorized into first, second and third line of defence (Noguchi and Niki, 1999; Baskin and Salem, 1997). These mechanisms of antioxidant defences are briefly illustrated in Figure 2.3(a-c).
First Line Antioxidant Defence

In first line antioxidant defence, an antioxidant is introduced to suppress the formation of free radicals (Figure 2.3(a)). Antioxidants that are involved in this mechanism defence are called preventive antioxidants. For instance, under oxidative stress state, an atom or molecule may break and form free radicals. These free radicals if not stopped, will result in a chain reaction that may cause further damage to the cell or tissue in the body. By introducing antioxidant at the initiation stage of free radicals, further oxidative damages can be hindered. The autoxidation of linoleic acid in a water-alcohol system and thiobarbituric acid (TBA) assay system were used to investigate the antioxidants’ potential in suppressing free radical initiation (Vimala, 2008).

Second Line Antioxidant Defence

The second line of antioxidant defence involves the scavenging of free radicals and also inhibition of chain initiation and propagation. Antioxidants under this category are known as radical scavenging antioxidants. These antioxidants are usually small
molecules (Figure 2.3(b)). In this line of antioxidant defence, antioxidants are introduced into the system to scavenge the available free radicals and also neutralize them to prevent the initiation and propagation of a chain reaction. Antioxidants are then quickly renewed into the system again and scavenge other free radicals. The antioxidant bioassays involved in evaluating the potential of an antioxidant in inhibiting free radical chain propagation are xanthine/xanthine oxidase (XOD) superoxide scavenging system, the DPPH free radical scavenging system, ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) (Vimala, 2008).

**Figure 2.3(b) Second line antioxidant defence mechanism - radical scavenging and inhibition of chain initiation and propagation** (A: antioxidant; R: radical)

**Third Line Antioxidant Defence**

The objective of the third line antioxidant defence mechanism is a repair mechanism to arrest the oxidative damages that may cause the degeneration of vitamins,
enzymes and interruption of the *de novo* pathways. *De novo* pathways form biomolecules from simple precursor molecules in the body which may act as antioxidant and fight against oxidative damages. The introduction of antioxidants into the system terminates the radical chain reaction. Antioxidants sacrifice themselves to compensate the unstable radicals with their own electrons, quenching the free radicals’ needs to ‘steal’ electrons from other molecules to stabilize themselves. Hence, antioxidants that play a role in this third line defence are known as terminating or sacrificial antioxidants and they are usually large molecules like enzymes and vitamins (Figure 2.3(c)). The β-carotene linoleate model system and tyrosinase inhibitory assay system are used to investigate antioxidants’ potential as a third line antioxidant defence agent (Vimala, 2008).

\[
\begin{align*}
\text{Sacrifice} & \quad A^\circ & \quad R^\circ & \quad A^\circ & \quad R^\circ & \quad A^\circ & \quad R^\circ \\
\text{Termination} & \quad R1^\circ + °R1 \rightarrow R - R & \quad A^\circ + °R \rightarrow A - R & \quad A^\circ + °A \rightarrow A - A & \quad A - R + °R \rightarrow A^\circ + R - R
\end{align*}
\]

**Figure 2.3(c) Third line antioxidant defence mechanism-sacrificial antioxidants and termination of oxidative stress progression** *(A: antioxidant; R: radical)*

However, no one antioxidant is specific for one antioxidant defence mechanism. The antioxidants involved in inhibition of free radical formation may also scavenge free radicals and sacrifice themselves to arrest the progression of oxidative damages. It was because the potential of an antioxidant was attributed to the additive and synergistic effects of combined phytochemicals and that explained why no antioxidant can play a single specific role in defending oxidative stress and damages (Liu, 2003).
2.8.3 Antioxidant and Cancer

The consumption of dietary antioxidants is associated with the prevention of oxidative damages thus promoting human health in general (Wang and Zheng, 2001). Many antioxidants compounds were naturally occurring in plants and they have gained the recognition as free radical and/or ROS scavengers (Gulcin et al., 2004).

The mechanisms of antioxidants in protecting against cancer was claimed to be involved in the early stages of cancer development. Schafer and Buettner, (2001) reported that antioxidants were used to change the redox environment and behaviour of cancer cells. It was also revealed that antioxidants may be potent in reducing the genetic instability of carcinoma cells and thus may be useful to improve the efficacy of cancer treatment (Reddy et al., 2001).

For example, vitamin C was reported to help regenerate vitamin E or α-tocopherol which is the major lipid soluble antioxidant found in cells. Vitamin E prevents the peroxidation of phospholipids in the cell membrane by donating its own single-electron hydrogen atom in the hydroxyl-group to the free radical, neutralizing the free radical before it triggers lipid peroxidation (Lampi et al., 2002). According to Davey et al., (2000), vitamin C itself is an enzyme cofactor, free radical scavenger and as an electron donor or acceptor. At non-toxic concentration, this vitamin enhances the cytotoxic effects of platinating agent, cisplatin against HeLa cells in vitro by stabilizing the tumour suppressor gene, p53 (Reddy et al., 2001).

Palozza et al., (2001) had claimed that β-carotene may inhibit ROS production in low concentrations but did not induce apoptosis in human colon adenocarcinoma
cells. However, this compound acted as a pro-oxidant and enhanced the production of ROS as well as inhibited the human colon adenocarcinoma cell growth at levels of \( \beta \)-carotene just above those seen in the non-supplemented human serum subjects (i.e. 2.5 to 20 \( \mu \)m).

Tea catechins were claimed to inhibit tumourigenesis (Clark and You, 2006) and induce apoptosis in pre-neoplastic cancer cells (reviewed in Yang et al., 2002). These polyphenols were also potential metal ion chelators and ROS trappers due to their polyphenolic structure thus hindering oxidative DNA damage (reviewed in Yang et al., 2002).

As reviewed in Rao and Rao, (2007), carotenoids are important as antioxidants by quenching ROS, inhibiting mutagenesis, reduce tumour development in vivo and reduce lipid peroxidation in low-density lipoprotein (LDL). For instance, lycopene and \( \beta \)-carotene were potential \( ^1 \)O\(_2\) and RO\(_2^\cdot\) quenchers (Kikugawa et al., 1997) and this was evident when Panaseko et al., (2000) demonstrated the antioxidant activities of lycopene and \( \beta \)-carotene in scavenging peroxynitrite in low-density lipoproteins.

Phenolic compounds especially flavonoids are also well-known for their antioxidant activity, capillary protective effect and also inhibitory effects against the development of cancer. According to Cushnie and Lamb, (2005), quercetin is the most potent scavenger of ROS and was reported to enhance the potential of endogenous antioxidants (Arts et al., 2004). These antioxidative potentials of quercetin are attributed to the presence of the catechol group in the B-ring and the OH-group at position 3 in the AC-ring (Heijnen et al., 2002).
With all these evidence, it is apparent that the usage of antioxidants may be beneficial when used in combination with certain types of chemotherapy and because of this; antioxidants have created new frontiers of hope for cancer prevention.

2.8.4 Antioxidant Bioassay Methods

It is difficult to attribute antioxidant capacity of a certain compound or a small group of components in a plant crude extract because the efficacy of the antioxidant activity depends on multiple factors such as concentration, the type of matrix, isomeric forms or the synergistic effects with other compounds or components. Hence, numerous methods have been developed in the hope of investigating the total antioxidant capacity of an extract, independent of the quantitative content of each individual compounds, where such activity might be attributed to.

These bioassay methods are preferred to be simple, inexpensive and precise in determining the effective activity of a single compound or of a complex crude extract (Almela et al., 2006). The methods used to evaluate antioxidant activity can be classified into three groups; the indirect methods, methods based on the usage of lipid oxidation metabolites and methods based on the radical scavenging activity (Table 2.2).
Table 2.2 The types of methods used to evaluate antioxidant activity

<table>
<thead>
<tr>
<th>Methods Used for Antioxidant Evaluation</th>
<th>Examples of Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indirect Methods</td>
<td>Ferric Reducing Antioxidant Power Assay (FRAP); Reducing Power Assay (Fe³⁺ reduction); Briggs-Rauscher Reaction (BRR); Determination of Total Phenolic Content; and Methyl Linoleate Assay</td>
</tr>
<tr>
<td>Based on Radical Scavenging Activity</td>
<td>Oxygen Radical Absorbance Capacity Assay (ORAC); 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) Assay (ABTS); Total Radical-Trapping Antioxidant Parameter Assay (TRAP); and 1,1-Diphenyl-2-picrylhydrazyl Hydrate Assay (DPPH)</td>
</tr>
<tr>
<td>Based on the Usage of Lipid Oxidation Metabolites</td>
<td>Thiobarbituric Acid (TBA); Ferric Thiocyanate (FTC); β-carotene Bleaching Inhibition Assay; and Rancimat</td>
</tr>
</tbody>
</table>

(Almela et al., 2006; Amarowicz et al., 2004; Dorman et al., 2003; Rahmat et al., 2003; Cervellati et al., 2002)

The groups of methods developed to evaluate antioxidant activity have their own advantages and disadvantages. The relevance of the indirect methods is clearly suggested by its name while on the other hand, methods based on radical scavenging activity are simple and quite effective. However, the advantage of these methods is dependent on the hydrophilic or lipophilic character of the crude extract tested (Wellwood and Cole, 2004; Lo et al., 2002). For methods based on the usage of lipid oxidation metabolites such as Rancimat, it requires the usage of specific instruments or otherwise they may show low selectivity (Schwarz et al., 2000).
Therefore, it is clearly seen that not a single method can be solely use to comprehensively predict the antioxidant efficacy of an extract or compound due to the complex nature of phytochemicals (Chu et al., 2000). Furthermore, the usage of more than one method is recommended because different methods of evaluation address different mechanism of action (Aruoma, 2003; Nuutila et al., 2003). A few examples of antioxidant activity evaluation methods’ mechanisms are briefly mentioned in the paragraphs below.

**β-Carotene Bleaching Inhibition Assay**

In the absence of an antioxidant, β-carotene undergoes rapid discolouration in this bioassay system. During oxidation, the hydrogen atom located on the carbon-11, between the double bonds, of the active bis-allyic methylene group of linoleic-acid is abstracted subsequently forming the pentadienyl free radicals (Frankel, 1998). These radicals then attack the highly unsaturated β-carotene molecules in its effort to reacquire the lost hydrogen atom. Consequently, the β-carotene molecules lose their conjugation and thus lose their characteristic orange colour and this process is monitored spectrophotometrically. However, the presence of an antioxidant may hinder the bleaching process of β-carotene because the antioxidant acts as a hydrogen-atom donor and compensate the loss of the hydrogen atom in the linoleic acid (Amarowicz et al., 2004).

**The FTC Assay**

The amounts of peroxide in the initial stages of oxidation during incubation are measured using the FTC assay (Gulcin, 2006a). In the initial stages of lipid peroxidation, the peroxide will react with ferrous chloride subsequently forming ferric ion. FTC complex is formed when the ferric ion produced earlier bond with
ammonium thiocyanate (Rahmat et al., 2003). The Fe$^{3+}$/thiocyanate complex is red in colour and detectable at 500 nm. The advantage of the FTC assay is that the thiocyanate ion is specifically bound for Fe$^{3+}$ only and forms the Fe$^{3+}$/thiocyanate complex. This complex gives out a single absorbance peak which is easily detected at 500 nm (Wong and Kitts, 2001). The oxidation of linoleic acid is retarded when antioxidants are present (Jayaprakasha et al., 2001).

**DPPH Free Radical Scavenging Activity Assay**

The evaluation of free radical scavenging effects of antioxidant substances in food systems was often done using the DPPH radical (Ozcelik et al., 2003). The DPPH assay is based on the reduction of DPPH radicals in alcoholic solution by antioxidants with hydrogen-donating affinity thus producing a non-radical form of DPPH-H in the reaction. The antioxidants reduced the stable DPPH radical (dark purple) to diphenyl-picrylhydrazine, a yellow-coloured substance.

The DPPH radical (DPPH°) gives full absorption at 517 nm but when reduced by an antioxidant (AH) or a hydrogen-donating radical species (R°), the absorption decreases thus indicating that a scavenging activity has taken place. The lower the absorbance obtained from the reaction mixture, the higher the free radical scavenging effects of the antioxidant investigated (Gulcin et al., 2006b). The DPPH radical reduction is as indicated (Brand-Williams et al., 1995).

\[
\text{DPPH}^\circ + \text{AH} \rightarrow \text{DPPH-H} + \text{A}^\circ \\
\text{DPPH}^\circ + \text{R}^\circ \rightarrow \text{DPPH-R}
\]
The use of DPPH radical has provided a simple and rapid way in determining the antiradical activities of antioxidants. It can accommodate a large number of samples and was sensitive in detecting natural compounds at low concentrations (Russo et al., 2005). The interaction between the DPPH radical and an antioxidant was reported to be dependent on the structural conformation of the antioxidant. The scavenging activity was seen to be corresponding with the number of available hydroxyl groups in the antioxidant (Brand-Williams et al., 1995). Thus it has been suggested that plant-derived antioxidants might act as prophylactic agents as the reduction-oxidation (redox) mechanism was implicated in the pathogenesis of human disorders (Aruoma, 2003).

The Reducing Power Assay

In this reducing power assay, the yellow colour of the reactive solution changes into various shades of green and blue, dependent on the reducing power of each extract tested. The Fe\(^{3+}\)/ferricyanide complex is reduced to the ferrous form, Fe\(^{2+}\), by reductants (i.e. antioxidants) and this is indicated by the formation of Perl’s Prussian blue, which can be monitored spectrophotometrically at 700 nm (Chung et al., 2002). This Fe\(^{3+}\)/ferricyanide system provides a sensitive ‘semi-quantitative’ determination of diluted polyphenolics in a redox reaction (Amarowicz et al., 2004). It can also serve as a significant indicator of an extract’s potential as an antioxidant (Gulcin, 2006a).

2.8.5 Determination of Total Phenolic Content

The crude estimation of phenolic compounds present in an extract can be done using the Folin-Ciocalteau reagent. When tested, the phenolic compounds engaged in a complex redox reaction with the phosphotungstic and phosphomolybdic acids in the
reagent (Wong et al., 2006). However, previous studies have shown that this assay is not specific towards polyphenols but also to any other substance that could be reduced by the Folin-Ciocalteau reagent (Escarpa and Gonzalez, 2001). Phenolic compounds with different number of phenolic groups respond differently to the reagent (Singleton et al., 1999).

2.8.6 Determination of Total Flavonoid Content

A spectrophotometric quantification of flavonoid using AlCl₃ solution was used in the present study. It has previously been utilized to determine the flavonoid content of propolis extracts (Chang et al., 2002). The antioxidant properties present can be determined by the presence of flavonoid with a certain molecular structure, especially those with a certain hydroxyl position and it is very much depended on its ability to donate hydrogen (H₂) or electrons to a free radical (Meda et al., 2005). However, this AlCl₃ method is specific only for flavones and flavonol and the total flavonoid content must be the sum of flavonoid content using the AlCl₃ method and also the 2,4-dinitrophenylhydrazine method which is specific for flavanone (Chang et al., 2002).

2.9 Human Papillomavirus

Human papillomavirus (HPV) is type A papovavirus. HPVs can cause warts and papillomas. These viruses are the only viruses in the Papovaviridae family that exhibit tumourigenic properties (Bonnez, 1997; Doyle, 1991).

Biological Properties

The HPV consists of a spherical protein coat that envelope the circular, double-stranded DNA genome. Their DNA genome is made up of approximately 7,900 base
pairs (Steenbergen et al., 2005). These viruses with 72 capsomeres were said to contain 360 copies of late gene-1 (L1) and probably 12 copies of late gene-2 (L2) (Modis et al., 2002).

**Typing of HPV**

To be identified as a new genotype, a HPV isolate must show a L1 sequence difference of more than 10% from any previously known HPV. In the same genotype, the discrimination among sub-types is between 2% to 10% and variants differ from each other by less than 2% in their nucleotide sequence (De Villiers et al., 2004).

**Classification of HPV**

HPVs are classified based on their biological niche, oncogenic potential and phylogenetic position and to date, 118 HPV genotypes have been identified (De Villiers et al., 2004). The HPV strains are classified as low-risk, intermediate-risk and high-risk types based on their association with cervical carcinoma and also with the associated precursor lesions (Munoz et al., 2003).

HPV types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 are the lower-risk HPVs and could cause non-cancerous genital warts whereas the oncogenic or high-risk types are HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, with types 26, 53 and 66 probably oncogenic. These oncogenic or high-risk HPV types were related to high grade cervical intraepithelial neoplasia (CIN) lesions and invasive carcinomas (Munoz et al., 2003).
High-risk HPV DNA was found in almost 90% of carcinoma of cervix and 30-50% of other anogenital squamous cancers (Munoz et al., 2006). The relationship with HPV was observed for all grades of intraepithelial lesions and the strength of association are most pronounced for the cancer-associated types such as HPV 16 and HPV 18. HPV 16 is accounted for about 50% while HPV 18 is responsible for about 20% of HPV-associated cancers and these two variants are particularly prone to persist and initiate cancers (Bosch and De, 2007).

2.9.1 HPV Oncoproteins

The HPV genome, which consists of approximately 7,900 nucleotides has eight overlapping open reading frames and is subdivided into three regions; the early region (E), late region (L) and the upstream regulatory region (URR) (Steenbergen et al., 2005). The HPV genome regions are illustrated in Figure 2.4.

![Figure 2.4 The schematic representation of the HPV genome](image)

Figure 2.4 The schematic representation of the HPV genome (Adapted from Munoz et al., 2006)
The early genes are responsible for encoding viral proteins that are involved in viral DNA synthesis and also cellular transformation and the genes are designated as E1, E2, E4, E5, E6, E7 and E8 while the late genes, L1 and L2, encode the proteins that are important for the formation of the capsid coat (Munoz et al., 2006). The URR section is a non-coding region (Munoz et al., 2006). The roles of HPV oncogenes are briefly presented in Table 2.3.

Table 2.3 An overview of the regions of the HPV genome

<table>
<thead>
<tr>
<th>Regions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Early Genes (E)</strong></td>
<td></td>
</tr>
<tr>
<td>E1 and E2</td>
<td>The E1 and E2 genes are important in basal DNA replication. The viral genome is replicated to about 100 copies and maintained for a varying periods in initially infected but replicating and competent host cells (Doorbar, 2005). E1 gene is responsible for genome maintenance and replication (Stoler, 2003) while E2, major transregulatory protein involved in transcription and replication of viral DNA (Kalantari et al., 1998). Correct segregation of genomes during cell division is facilitated by these genes (You et al., 2004).</td>
</tr>
<tr>
<td>E4</td>
<td>The E4 gene product is involved in the maturation and release of new HPV particles. When E4 interrupts cytoplasmic cytokeratin, koilocytes appear (Stoler, 2000), facilitating the release of new viral particles (Wang et al., 2004b).</td>
</tr>
<tr>
<td>E5</td>
<td>A small hydrophobic protein on the cellular membrane and it is involved in stimulating the HPV growth and cell transformation (Stoler, 2000) and viral DNA amplification (Fehrmann et al., 2003).</td>
</tr>
</tbody>
</table>
E6 and E7 are critical genes in HPV replication. These genes have the ability to induce cell proliferation and immortalization and eventually transformation of host cells into malignant cells (Munger et al., 2004). They react with cellular proteins and this interaction is best characterized by the binding of p53 and pRb tumour suppressor genes which are often found mutated in various human cancers (Munoz et al., 2006). E6 gene binds to p53 and immortalizes its function while E7 targets the pRb gene. The binding of E7 to pRb activates the transcription factor, E2F which encourages DNA replication (Munger et al., 2004). Constant activity of E6 and E7 results in genomic instability, increased oncogene mutations, retardation of cell growth and consequently cancer (Duensing and Munger, 2004).

Late Genes (L)
L1 and L2
The L1 and L2 genes are responsible for coding major and minor capsid proteins (Steenbergen et al., 2005).

Upstream Regulatory Region (URR)
This region contains cis-elements required for gene expression regulation, genome replication and viral particle packaging (Munoz et al., 2006).

2.9.2 The E6 Proteins of HPV
HPV types 16 and 18 were reported to exert immortalizing and transforming abilities in human cells (Munger et al., 2001) through a biological property that is confined to two small open reading frames (ORF) that encode the E6 and E7 proteins; which help promote the survival and replication of HPVs (Hall and Alexander, 2003; zur Hausen, 2000). E6 was one of the first genes to be expressed during HPV infection and the uncoupling of E6 gene from its normal function during the HPV life cycle allows this gene to act as an oncogene (Fehrmann and Laimins, 2003; Flores et al., 2000).

E6 is a 150 amino acid protein and is characterized by four Cys-X-X-Cys motifs which are believed to be involved in zinc binding. The importance of these motifs
has been associated in functions such as transcriptional activation, transformation, immortalization and association with cellular proteins (Fehrmann and Laimins, 2003).

**Biological Functions of E6**

E6 is a multi-functional protein that interacts with cellular proteins, mediates immortalization of primary cells and transformation of the established cell lines, tumourigenesis and apoptosis. E6 also modulates cell transcription process and telomerase activity.

i) p53 Binding and Degradation

The inactivation of the p53 gene by E6 was suggested as a mechanism where this oncoprotein actually promotes the growth and proliferation of the viral cells. It is known that over-expression of p53 would induce the ubiquitin-dependent p53 degradation resulting in cell cycle arrest or apoptosis that subsequently inhibits viral replication. E6 proteins of high-risk HPV counteract this effect by binding to and inactivating the tumour suppressor protein, p53 causing the hindrance of apoptosis.

E6 proteins induce ubiquitination and proteolysis of the p53 through interaction with the E6-associated protein, E6-AP (Fehrmann and Laimins, 2003). The same E6/E6-AP complex was suspected to also function as an ubiquitin-protein ligase that targets the degradation of some cellular proteins as well. It was previously claimed that the degradation of p53 only takes place in the presence of both E6 and E6-AP (Daniels et al., 1998).
ii) Telomerase Activity

In addition to p53, E6 proteins also interact with telomerase which is responsible in the telomeric DNA replication. Telomerase is short DNA repeat units present at the ends of the chromosome and these short DNA repeat units are important in protecting the chromosome ends from damage and degradation (Greider and Blackburn, 1996). Telomerase activity prevents the shortening of the DNA telomere and Klingelhutz et al., (1996) had investigated the link between E6 expression, immortalization and telomerase activation.

Activation of telomerase is a critical step in cellular transformation (Elenbaas et al., 2001). It was claimed that the expression of E6 activates telomerase in human foreskin keratinocytes and mammary epithelial cells. However, no telomerase activity was observed in cells expressing E7 alone (Klingelhutz et al., 1996). Consistent with this observation, Oh et al., (2001) and Veldman et al., (2001) also detected significant telomerase activity in human foreskin keratinocytes. In addition to that, Oh et al., (2001) observed that cells expressing both E6 and E7 exerted greater telomerase activity and became immortalize and E7 was suspected to augment E6-mediated telomerase activation.

iii) Cellular Immortalization and Transformation

The immortalization property is only shared among the high-risk HPV E6 and E7 genes. This activity may exist in low-risk HPVs but it is weak or not active (Munger et al., 1989). E6 is able to independently immortalize human cells but the coupling of E6 and E7 creates a synergistic effect which subsequently increases the transformation efficiency (Fehrmann and Laimins, 2003). It was suggested that both E6 and E7 genes are crucial and cooperate to induce immortalization of human
genital keratinocytes. Nevertheless, E6 on its own can induce immortalization of certain cells (Wazer et al., 1995). For instance, E6 can immortalize human mammary epithelial cells (Klingelhutz et al., 1996) while the coupling of E6/E7 is required to cause the immortalization of primary human oesophageal cells (Zhang et al., 2007).

It was often observed that E6 did not act alone in the induction of cellular transformation. On some attempts, E6 was often coupled with either E7 or ras oncogene for that purpose. Liu et al., (1994) reported the coupling of E6 protein of HPV type 16 with activated ras oncogene to induce transformation of baby rat kidney cells while the combination of E6 and E7 was found to convert the phenotype of differentiated primary human keratinocytes (Halbert et al., 1992). The transformation of human embryonic kidney cells was attempted with E6 and E7 combination (Nakagawa et al., 1995). In another study, it was revealed that HPV type 18 E6 alone was sufficient to alter differentiation of human keratinocytes (Hudson et al., 1990).

iv) Transcriptional Activation
The growth promoting function of oncogenes is mediated by transcriptional activation function. E6 was demonstrated to be involved in both transcriptional activation (Desaintes et al., 1992) and repression (Etscheid et al., 1994) activities. E6 transcriptional activation ability was observed in many promoter genes such as telomerase hTERT (Veldman et al., 2001), VEGF (Clere et al., 2007; Lopez-Ocejo et al., 2000), hepatitis B virus enhancer I (Lee et al., 1999), transforming growth factor-β1 (TGF- β1) (Dey et al., 1997), adenovirus E2 promoter (Shirawasa et al., 1994; Sedman et al., 1991) and oncogene promoters like c-myc (Kinoshita et al.,
E6 was also involved in the transactivation of the early HPV promoter in the URR (Gius et al., 1988).

Etscheid et al., (1994) had demonstrated HPV 16 E6 transcriptional repression in cervical cancer-derived cells, C33a and osteosarcoma cell line, Saos-2. C33a expresses mutant p53 while Saos-2 was lack of p53. Hence this had suggested that the HPV 16 E6-dependent repression activity was not mediated by p53. Transcriptional suppression by E6 was reported in Degenhardt and Silverstein, (2001) where HPV type 18 E6 inhibits Gps2-mediated transcriptional activation activity by inducing Gps2 degradation. Gps2 is a protein partner for HPV E6 proteins.

v) Apoptosis

Apoptosis eliminates the survival of radiation damaged, aberrant growing and virally infected cells in an organism to help maintain its survival. However, it is intuitive to propose that apoptosis is suppressed since the E6 protein binds and degrades p53. It was reviewed in Tristam and Fiander, (2007) that the integration of HPV into the host genome will subvert the apoptotic response to irreversible DNA damage. High-risk HPV E6 was reported to inhibit both p53-dependent and p53-independent apoptosis (Jackson et al., 1998) and this effect is consistent with the findings earlier that claimed HPV type 16 E6 protein modulates apoptosis through the p53-dependent and p53-independent pathways (Xu et al., 1995). Apoptosis can also be triggered through caspase activation and mitochondrial pathway as researched by Liu et al., (2007).
Nonetheless, previous studies have shown that the HPV 16 E6 proteins were found to both increase and decrease cell programmed death in various cell lines in response to various apoptosis inducing agents. The E6 protein increased apoptosis of immortalized human keratinocytes and human mammary epithelial cells in the presence of DNA damaging agents such as mitomycin-C, taxol, etoposide and tamoxifen (Liu et al., 2007; Seewaldt et al., 2001).

2.9.3 HPV and Cervical Cancer

Cervical cancer is the second most common cancer worldwide, with an annual incidence of 500,000 with a mortality rate of 50% (Parkin et al., 2001). At least 15 types of HPVs were reported to be associated with cervical carcinoma and high grade CIN (Kirwan and Herrington, 2001) and it seems like more than 99% of cervical carcinoma is associated with HPV infection (Bosch and De, 2007; Monsonego, 2005) and etiologically linked to more than 50% of other anogenital cancers (zur Hausen, 2001).

HPV infection of the cervix is among the most common sexually transmitted infections (Woodman et al., 2007). Approximately 99% of human cervical cancers harbour high-risk types HPV DNA, with type 16 being the most prevalent, followed by types 18, 31, 33 and 45. According to Miller et al., (2000), cervical cancer is both preventable and curable. With the introduction of early detection and cervical cancer control programmes, 80% of cervical cancer incidence and mortality rates were reduced in some developing countries.

While an estimated 470,000 new cases was reported worldwide in year 2000 (Pecorelli et al., 2003), cervical cancer was the second most frequent cancer among
women, after breast cancer in Malaysia. In year 2002, a total of 1,715 confirmed cases of cervical cancer which constituted a 12.0% of total female cancers in Malaysia and increased to 12.9% of total female cancer cases diagnosed in year 2003. The National Cancer Registry in Malaysia has reported that 21,464 cancer cases were diagnosed in Peninsular Malaysia and 2,282 cases were registered in East Malaysia in the year 2003 (Lim et al., 2003; 2004).

Therefore, early detection and prevention are important because early stages of cervical cancer often do not cause pain or other symptoms. Cancer of the cervix is easily detected using the Papanicolaou’s smear (Pap smear) which was discovered back in 1940’s by George Nicholas Papanicolaou. Although the cervical cancer early detection programme was introduced about four decades ago in Malaysia, yet only some 33% of women went for their regular checks (Othman, 2003).

The association between HPV and CIN has lead to the assumption that HPV infection is the key to carcinogenesis of cervical cancer (Carozzi et al., 2000). The carcinogenesis of cervical cancer is simplified in Figure 2.5.

![Figure 2.5 The brief illustration of carcinogenesis of cervical cancer](image)

(Adapted from Moscicki et al., 2006)
The persistent expression of HPV DNA in malignant cells was demonstrated in several human cervical cancer-derived cell lines. Convincingly, six cell lines were found to contain HPV DNA sequences when investigated using HPV 6, 11, 16 and 18 DNA probes. Two of the cell lines, HeLa and C4-I had previously shown to carry HPV 18 DNA sequences, which were integrated, into the host cell.

HeLa cells carried about 10-50 integrated copies of HPV type 18 per cell while C4-I cells were estimated to have one copy of the same HPV type. The other two cell lines, CaSki and SiHa were intensely harboured with HPV type 16 DNA. It was estimated that the CaSki line contained more than 500 copies of HPV and while the SiHa line contained approximately ten copies of DNA (Fletcher et al., 1991; Schwarz et al., 1985).

Wu et al., (2006) assessed the HPV types association with cervical cancer using 1,010 cervical cancer samples collected from inpatients in China. Among them, 475 cases had cervical squamous cell carcinoma, 66 patients had carcinoma in situ (CIS), 262 cases were CIN patients, 139 patients were infected with cervicitis and the rest were healthy individuals. In this study, 152 patients were typed for HPV. Overall results showed that most of the subjects were tested positive for HPV type 16 (79.6%) and HPV type 18 was uncommonly found among the cervical cancer patients.

In an unrelated study, a cohort of 213 women with cytologically abnormalities was recruited to assess the prevalence and distribution of HPV infections contributed by different types of HPVs. Of these, 41 cases were atypical squamous cells of undetermined significance (ASCUS), 17 cases of atypical glandular cells of
undetermined significance (AGUS), 96 cases were low-grade squamous intraepithelial lesion (LSIL), 55 cases of high-grade intraepithelial lesions (HSIL), 3 has CIS and one case of invasive carcinoma (Gargiulo et al., 2007).

According to Gargiulo et al., (2007), in all cervical cancer samples, the HPV DNA of type 16 and 52 were most prevalent with the detection of 61.2% cases with HPV type 16 and 6.1% cases with HPV type 52. HPV type 52 was consistently found in CIN-I patients while HPV type 16 was predominantly found in other CIN and cervical cancers. HPV type 18 DNA was found in 12.9% cases while 9.6% cases was reported with HPV type 31. The findings revealed that HPV DNA was detected in 84.9% of the cases in this cohort study.

The prevalence and distribution of HPV type 16 reflected in these studies had strengthened the association of high-risk HPV infection with the development of cervical cancer.

2.10 Immunocytochemistry

Immunocytochemistry (ICC) is a branch of immunology concerning with the identification of a tissue constituent in situ based on a specific antigen-antibody interaction (Van Noorden and Polak, 1983). Immunocytochemical staining has been used extensively to detect viral antigen as it is a sensitive, specific method of localizing antigens with the use of labelled antibody. ICC was first introduced by Coons et al., to detect pneumococcal antigens in the livers and spleens of experimentally infected mice using fluorescein labelled anti-pneumococcal antibody (Coons et al., 1942; Coons et al., 1941).
Polyclonal antibodies were widely used in the early methods of immunochemical staining although these antibodies were often in short supply and sometimes had significant variations among lots. When monoclonal antibody was introduced, it has helped overcome the polyclonal antibody issues and has greatly improved the specificities of the immunochemical staining method. There are two basic methods of immunochemical staining; the direct method and the indirect method.

**Direct Method**

The direct method is the older technique where an enzyme-labelled primary antibody is allowed to react with the antigen in the tissue. A single virus-specific antibody is directly labelled with an indicator, such as fluorescein or alkaline phosphatase (Lakeman, 1997). The formation of a coloured complex at the end of the assay concludes the reaction sequence. However, due to the insufficiency of sensitivity, this method is no longer favoured (Boenisch, 2006b).

**Indirect Method**

The indirect method requires two antibodies, a virus-specific antibody to bind with the antigen while the other one is an anti-species antibody labelled with the indicator (Lakeman, 1997). The indirect method is much preferred as it is much more versatile compared to the direct method because primary antibodies from the same species can be utilized with the same conjugated secondary antibody. The indirect method has two branches; the two-step indirect method and the three-step indirect method (Boenisch, 2006b).

In the present study, the three-step indirect method is used. Briefly, there are three incubations prior to colour development at the end of the assay. Firstly, a non-
conjugated specific primary antibody is introduced to the viral antigen of the test samples. Then, a biotinylated secondary antibody is directed against the primary antibody before allowing further incubation with horseradish peroxidase (HRP) enzyme-conjugated avidin or streptavidin forming a biotin-streptavidin HRP complex (Boenisch, 2006b; Forghani and Hagens, 1995).

Bound antibody labelled with HRP is detected using a substrate chromogen such as 3,3’-diaminobenzidine tetrahydrochloride (DAB) whereby its oxidized form forms an insoluble, non-diffusing dark brown precipitate polymer that settles at the site of antigen that can be easily visualized under the light microscope (Lakeman, 1997; Pearse, 1972). Bratthauer (1994) had illustrated the three-step indirect method as featured in Figure 2.6.

![Figure 2.6 Diagram illustrating the three-step indirect labelled avidin binding (LAB) procedure](Adapted from Bratthauer, 1994)

Avidin

Peroxidase-Labelled Avidin

Biotinylated Secondary Antibody

Primary Antibody

Tissue Antigen

(immunoglobulin; long carbon arm extension; biotin; avidin; peroxidase)
2.11 The Liliaceae Family

The Liliaceae family or more commonly known as the lily family consists of over 200 genera, about 3,000 species and are mostly represented by herbs or occasionally woody plants; rhizomes, bulbs, corms or other fleshy structures (Benson, 1979). The members of this family have radical or cauline leaves with bisexual flowers than are often actinomorphic. The flower has a six segment perianth with six stamens. Generally, the Liliaceae members have a superior three-loculate ovary and its fruit is a capsule or berry. Its roots are from a rhizome, corm or bulb (Hsuan, 1969). Alliaceae is one of the family’s many segregate and a few common members of this Alliaceae segregate are namely Allium cepa L. (onion), Allium sativum L. (garlic) and Allium porrum L. (leek) (Benson, 1979).

2.11.1 Allium Genus

This genus consists of about 700 biennial and perennial herbs species that has a significant pungent odour when crushed. Majority of Alliums are known as wild plants with little economical value and they are common in open fields, scrubs and deserts. Bulbs are a common characteristic of the members of this genus and they are often covered by membranous or fibrous outer scales (Dahlgren et al., 1985).

These plants also have underground storage structures such as rhizomes while some others may have storage roots. They have foliage leaves with scapes that are angular and bear umbel-like inflorescence of flowers, with short or long pedicels that are attached to the underground stem with long sheathing bases (Dahlgren et al., 1985). Alliums have petal-like perianth, which are free from one and another to below the middle. It always has six stamens with a superior ovary, which sits on top of the
flower parts. It has an umbel inflorescence with buds surrounded by a single spathe (Jones and Mann, 1963).

**The Distribution of *Allium* spp.**

*Allium* is the largest genus in the widely distributed Alliaceae family (Dahlgren *et al.*, 1985). According to Hedrick, (1972), Hippocrates said that *A. cepa* L. or better known as onion was already a common consumable back in 430 B.C. and it is a native to Asia and cultivated in Egypt, Japan, Europe, America, France, Mexico and Southeast Asia (Uhl, 2000). As for *A. fistulosum* L., or better known as spring onion was said to be originated from Far East (Hyam and Pankhurst, 1995) and introduced into England in 1629 and is widely used throughout Southeast Asia. The garden leek, *A. porrum* L. has been known throughout the Middle Ages (Hedrick, 1972).

The commoner, *A. sativum* L. or garlic probably originated from central Asia (Hutton, 2000) and has been cultivated since earliest time in most parts of the world. It was a well known native of the Western Tartar plains, later transported to other parts of the world especially Asia, Europe and North Africa. Garlic was brought into China in between 140 and 186 B.C (Hedrick, 1972). Garlic was mentioned in the Bible (Bergner, 1996) and the Talmud (Moyers, 1996). *Allium ursinum* L., a resemblance of garlic is also known as ramson. This herb was reported by Hedrick, (1972) as an origin of Europe and Northern Asia. The Chinese chives, *A. tuberosum* Rottl.ex Spreng is believed to be native to South East Asia (Floridata (2003), accessed 21 April 2008).
Domestic Usages of *Allium* spp.

The *Alliums* are commonly valued for food, medicine, garden ornamentals, and range plants and as weeds. Onions, garlic, leek, chives and Welsh onions are some of the most commonly valued *Alliums* (Table 2.4). Both onions and garlic can be used to treat disorders such as asthma, arthritis, arteriosclerosis, chicken pox, the common cold, diabetes, malaria, tumours, and heart problems (Uhl, 2000; Hedrick, 1972; Jones and Mann, 1963)

**Table 2.4 The domestic Alliums and its usages**

<table>
<thead>
<tr>
<th>Common Names</th>
<th>Scientific Names</th>
<th>Domestic Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onion, shallot, potato onion, bawang merah, yan chong</td>
<td><em>Allium cepa</em> L.</td>
<td>Seasoning, canned food, pickles</td>
</tr>
<tr>
<td>Welsh onion, Japanese bunching onion, spring onion</td>
<td><em>Allium fistulosum</em> L.</td>
<td>Seasoning, fresh consumption</td>
</tr>
<tr>
<td>Garden leek, kurrat, great headed garlic</td>
<td><em>Allium porrum</em> L.</td>
<td>Vegetables for fresh consumption, seasoning</td>
</tr>
<tr>
<td>Garlic, bawang putih, suan tou, kratien</td>
<td><em>Allium sativum</em> L.</td>
<td>Seasoning</td>
</tr>
<tr>
<td>Chinese chives, kow choi, kuchai</td>
<td><em>Allium tuberosum</em> Rottl. ex Spreng</td>
<td>Vegetables, seasoning</td>
</tr>
<tr>
<td>Ramson, wild garlic, bear's garlic</td>
<td><em>Allium ursinum</em> L.</td>
<td>Culinary herb</td>
</tr>
</tbody>
</table>

(Uhl, 2000; Hedrick, 1972; Jones and Mann, 1963)

**Phytochemicals of *Allium* spp.**

The major chemicals found in *Allium* spp. are carbohydrates, fats, proteins, minerals and vitamins. A high content of water was also reported in the bulbous species of
this genus (e.g. *A. sativum* L. is mainly composed of 56% to 68% of water). *Allium tuberosum* Rottl. contains folic acids and both vitamin A and C (Uhl, 2000; Lawson, 1996; Watt and Merrill, 1950). Trace amount of germanium, molybdenum and selenium found in garlic had increased its therapeutic value. A Japanese doctor has verified that germanium has anti-cancer properties (Kato, 1973). Selenium protects against tumourigenesis (Dorant *et al*., 1993) and also inhibits both initiation and post-initiation phases of chemical carcinogenesis in DMBA-induced mammary tumour in rats (Ip and Lisk, 1995).

It was reported that *A. cepa* L. have 0.01% to 0.015% of volatile oil that gives out a dark brown colouring which mainly consists of sulfur compounds. When garlic is crushed, cut or rehydrated, essential oils are formed through enzymatic reactions and an amount of 0.1% to 0.25% of essential oil is believed to be produced. On the other hand, the presence of non-volatile compounds such as acid amides, carbonyl, thioether and isothiocyanate compounds gives the *Allium* vegetables its pungency. The flavour similarities and differences among the members of this genus is influenced by the ratio of volatiles to non-volatiles content and this explains the differences in flavour among onions, chives, garlic, shallots and leeks (Uhl, 2000).

### 2.11.2 Bioactive Compounds of *Allium* spp.

The health beneficial effects of *Alliums’* were attributed to the especially high levels of organosulfur compounds and also the presence of other bioactive compounds such as steroidal saponins, sterols, thiols and prostaglandins (Kik *et al*., 2001). The biologically active compounds in the *Allium* vegetables can be categorized as sulfurous compounds and non-sulfurous compounds. The non-sulfurous compounds consist of flavonoids, prostaglandins, oligofructans, sterols and steroid saponins.
Sulfurous Compounds

The organosulfur compounds that are present in the *Allium* vegetables are either water- or lipid-soluble (Bianchini and Vainio, 2001) and it is believed that the beneficial effects of *Alliums* are credited to its high content of the organosulfur compound, S-alk(en)yl-cysteine sulfoxide, which is the volatile precursor of this genus, and its derivatives (Kik *et al.*, 2001). The main organosulfur compounds found in the *Allium* vegetables are illustrated in Figure 2.7.

The volatile sulfur compounds in the *Allium* vegetables are not present as such in intact cells. When the cells rupture, enzymatic reactions catalyzed by the allinase enzyme take place between the volatile precursors and sulfonic acid, producing different thiosulfinates and related sulfonic acid-derived compounds (Lancaster and Shaw, 1989). Thiosulfonates are transformed into thiosulfinates, thiols, thiophenes, sulfur dioxide, cepaenes and mono-, di-, tri- and tetrasulfides in the non-enzymatic degradation pathways (Delaquis and Mazza, 1998). An important thiosulfinate, allicin (diallyl thiosulfinate) is believed to be the element that gives that garlicky odour in the *Allium* vegetables, especially in garlic (Lawson, 1998).

i) Allicin

Allicin is the element that gives the typical garlic odour. Alliin, an odourless compound is converted into allicin through enzymatic reactions which is induced by the alliinase enzyme when garlic is crushed, cut or chopped (Block, 1985). Figure 2.8 shows the formation of allicin from alliin. Allicin is unstable and is easily converted into ajoene and other sulfides such as mono-, di- and trisulfide (Block, 1985).
Figure 2.7 The water- and lipid-soluble organosulfur compounds in *Allium* vegetables (Adapted from Tapiero *et al*., 2004)
Allicin is soluble in organic polar solvents but has low solubility in water. The half-life of pure allicin in water and 1mM of citric acid is 30 and 60 days respectively but the half-life of allicin will decrease to 16 hours if there is no solvent (Lawson, 1993). Allicin was reported to exert immune-stimulatory and anti-tumour properties in both human peripheral blood mononuclear cells (PBMC) and mouse splenocytes (Patya et al., 2004) and anti-microbial activity against honey bees pathogens (Aronstein and Hayes, 2004).

![Figure 2.8 The formation of allicin from alliin](Adapted from The Chemical Heritage Foundation, accessed on 21 April 2008)

**Flavonoids**

Flavonoids are water-soluble pigments that help protect the cells from damages caused by radiation, preventing the occurrence of multistage carcinogenesis in experimental animals and block the cells from synthesizing prostaglandins (Abdulla and Gruber, 2000). In *Alliums*, the few commonly found flavonoids include quercetin glucosides, 3-rutinoside (rutin), 3-rhamnoside (quercitrin), 3-glucosides of kaempferol, isorhamnetin-4’-glucoside and eight anthocyanins. These flavonoids have a wide array of biochemical functions and are potential antioxidant agents as they scavenge superoxide, hydroxyl and peroxyl radicals and also hamper chained lipid peroxidation reactions (Abdulla and Gruber, 2000; Mazza and Miniati, 1993).
i) Quercetin

Quercetin, a common dietary flavonoid, is found mostly in onions and plays a major role in complementing the many medicinal activity of *A. cepa* L. (Figure 2.9). Quercetin has been shown experimentally to have numerous benefits on the body. This compound is the most potent free radical scavenger (Cushnie and Lamb, 2005), inhibits xanthine-oxidase (Zhu *et al*., 2004) and reduced hepatic lipid peroxidation in mice (Panda and Kar, 2007).

Quercetin also exhibits anti-HIV activity (Kashiwada *et al*., 2005). It has also been shown that quercetin derivative can induce Epstein-Barr virus early antigen (EBV-EA) inactivation without cytotoxicity on Raji cells (Iwase *et al*., 2001). Anti-inflammatory and anti-proliferative properties of quercetin were also reported in Orsolic *et al*., (2004) and Rahman, (2002).

![Chemical structure of quercetin](image)

**Figure 2.9 Chemical structure of quercetin** (Adapted from Kim *et al*., 2005)

However, when quercetin was given intraperitoneally to mice, it was reported that it had helped increase the growth of injected melanoma cells (Drewa *et al*., 2001) and this suggests that caution has to be exercised.
Sterols and Steroid Saponins

The amount of sterol derivatives was reported to be lower in the bulbs as compared to the leaves of the plants. It was reported that there is 0.1% of saponin in leeks, 0.021% in garlic and 0.095% in onions (Smoczkievicz et al., 1982). 2.7% of free sterols and sterol esters, 1.7% of sterol glycosides and 0.8% of acetyl sterol glycosides were found in the leaves of an onion plant (Hanley and Fenwick, 1985).

Tuberoside was discovered in *A. tuberosum* L. (Zou et al., 2001) and chinenoside II, chinenoside VI and neomacrosteremonoside D were isolated from *Allium chinense* L. (Jiang et al., 1999; Peng et al., 1996). Four furostanol saponins were isolated from *A. cepa* L. (Yuan et al., 2008) while three saponins (i.e. minutoside A, minutoside B and minutoside C) and two known sapogenins, alliogenin and neoagigenin, were isolated from the bulbs of *A. minutiflorum* Regel. (Barile et al., 2007). (25R, S)-5ct-Spirostan-3fl-ol tetrasaccharide and (25R)-3fl-hydroxy-5ct-spirostan-6-one, di- and tri-saccharides from *A. chinense* L. bulbs showed inhibitory activities on both cAMP PDE and/or Na⁺/K⁺ATPase (Kuroda et al., 1995).

Oligofructans

The storage life of bulbs of the *Allium* species depends on the content of fructans found in them. Fructans are water-soluble carbohydrates and 35% to 40% dry weight of an onion bulb consists of fructans (Suzuki and Cutcliffe, 1989; Darbyshire and Henry, 1978). The benefits of these oligofructans and oligosaccharides when ingested include the reduction of toxic metabolites, pathogenic and autogenous diarrhea prevention, treating constipation, in reducing the level of serum cholesterol and blood pressure and anti-cancer effects (Tomomatsu, 1994; Hideka et al., 1986).
Prostaglandins

*Allium cepa* L. has been suggested to be a good source of prostaglandins (Al-Nagdy *et al.*, 1986; Attrep *et al.*, 1980) and as to date, they have managed to isolate and characterized prostaglandin A₁, A₂, B₁, E₁, F₁α, F₂α, D₂, E₂ and 6-keto-prostaglandin F₁α from the *Allium* plants (Ali *et al.*, 1990; Ustunes *et al.*, 1985).

2.11.3 The Health Benefits of *Allium* spp.

*Alliums* have been widely used in both traditional and folk medicine for their therapeutic properties and in the recent years, the availability of modern scientific techniques and applications has helped discover more benefits of these *Allium* vegetables. The health benefits of these *Alliums* were associated with the presence of bioactive principles in these plants such as organosulfur compounds, flavonoids and steroidal saponins.

Cardiovascular Disease

S-methylcysteine sulfoxide (SMCS), an organosulfur compound found in *A. cepa* L. exerted definite hypolipidemic effects by lowering the total cholesterol and both LDL and very low-density lipoprotein (VLDL) in high cholesterol diet-fed animals (Kumari and Augusti, 2007). By playing antioxidant, quercetin is thought to inhibit the biosynthesis of cholesterol (Glaber *et al.*, 2002). Other than that, quercetin contributes to the vasodilation of large or small arteries, including coronary arteries that help eases the risk of heart attack (Ibarra *et al.*, 2002; Perez-Viscaino *et al.*, 2002).

\[ \gamma \text{-glutamylcysteine, a natural angiotensin-I converting enzyme (ACE) inhibitor found in garlic help reduces blood pressure by inhibiting ACE (Sendl et al., 1992).} \]
Generally, patients with high blood pressure that consume a regular dose of garlic will experience a drop of 12.0 to 30.0 mm in the upper blood pressure and 7.0 to 20.0 mm in the lower blood pressure (Weiner and Weiner, 1994). The anti-hypertensive properties of garlic were also reported by Al-Qattan et al., (2001).

**Anti-bacterial, Anti-fungal and Anti-viral**

The naturally-occurring diallylsulfide (DAS) and diallyl disulfide (DADS) in garlic, Chinese leek and onion were shown to exert effective anti-bacterial activity against a few strains of *Aspergillus* spp., *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) (Tsao and Yin, 2001). DAS and DADS were also believed to be responsible for the inhibition of the growth of *Helicobacter pylori*, an ulcer-forming microorganism in order to help prevent gastric ulcers (Chung et al., 1998).

*Allium porrum* L. is rich in saponins and had demonstrated anti-fungal activity when tested against *Fusarium culmorum* (Carotenuto et al., 1999). Fistulosin (octadecyl 3-hydroxyindole), a novel anti-fungal compound in *A. fistulosum* L. (spring onion) showed high anti-fungal activity against *Fusarium oxysporum* (Phay et al., 1999). The anti-fungal activity of *A. fistulosum* L. was once again proven in another study with *A. cepa* L. and *A. sativum* L. (Pyun and Shin, 2006). Minutoside B, a saponin isolated from *A. minutiflorum* Regel exerted anti-fungal activity which was comparable with common natural antibiotics and synthetic fungicides (Barile et al., 2007).

Garlic was reported to exert anti-viral activity against a several genera of viruses such as human cytomegalovirus, influenza B, *Herpes simplex* type I and type II,

**Anti-diabetic**

Another study had reported that the hypoglycaemic potency of garlic was attributed to its sulfur compounds, mainly allicin (Chang and Johnson, 1980) that probably involves either direct or indirect stimulation of insulin secretion (Carson, 1987). The lowering of serum glucose, triglycerides, cholesterol, aspartate amino transferase (AST), alanine amino transferase (ALT), uric acid, urea and creatinine levels were seen in diabetic rats treated with garlic extracts (Eidi *et al*., 2005). Quercetin in *Allium* also exerted anti-diabetic potential in streptozocin-induced diabetic rats (Vessal *et al*., 2003).

**Anti-platelet Aggregation**

*Allium cepa* L., *A. sativum* L. and *A. ursinum* L. have been known to inhibit platelet aggregation and thromboxane A$_2$ (TXA$_2$) formation (Moon *et al*., 2000; Carotenuto *et al*., 1996; Ali, 1995). The human platelet aggregation *in vitro* properties of onion and garlic was also demonstrated in Pierre *et al*., (2005). Ajoene, found in garlic extracts exerted its potential in inhibiting platelet aggregation and formation of platelet thromboxane thus increasing fibrinolytic activity (Steiner and Lin, 1998; Apitz-Castro *et al*., 1986).

**2.11.4 Allium and Cancer**

In general, the consumption of *Allium* vegetables has lowered the risk of various cancers. An independent research in China showed that the increasing intake of *Alliums* can reduce the risk of stomach cancers and this effect was obviously seen in
garlic, scallions and Chinese chives (You et al., 1989). Another case-control study done on a population basis in Shanghai has proved that the intake of Allium vegetables including onions, scallions, garlic, chives and leeks had significantly lowered the risk of prostate cancer (Hsing et al., 2002).

Bianchini and Vainio, (2001) had associated the consumption of A. cepa L. with the overwhelming inverse correlation with the risk of stomach cancer. Shon et al., (2004) investigated the beneficial effects of different onion variants for their antioxidant and anti-mutagenic activities and found that these activities were related to their phenolic and flavonoid contents. Quercetin, extracted from A. cepa L. is another important inhibitor of cell growth and this compound exerted apoptotic activities against both human breast cancer cell line, MCF-7 (Choi et al., 2001) and human colon cancer cell line, HT-29 (Kim et al., 2005). Quercetin also provides protection against oxidative stress (Myhrstad et al., 2002) and lipid peroxidation (O’Reilly et al., 2001).

*Allium sativum* L. was found to be a potential anti-carcinogenic agent and its potential was claimed to be attributed to its high content of organosulfur compounds. Liu et al., (1992a) found that a diet consisting of 2-4% of garlic will delay the growth of breast cancer. It was shown to help reduce the number of tumours by 56% in a 20 week-study of rats fed with 2% of garlic powder compared to control fed rats. In the same study, Liu et al., (1992b) discovered that *A. sativum* L. also helps to increase the glutathione-S-transferase enzyme level in the body thus increasing the liver’s ability to detoxify carcinogens and other dangerous chemicals.
12-keto-porrigenin and 2,3-seco-porrigenin are sapogenins isolated from *A. porrum* L. and these compounds had demonstrated anti-proliferative activities against three murine cell lines, WEHI 164, J774 and P388 and human melanoma IGR-1 cells (Carotenuto *et al*., 1999). *Allium ursinum* L. extracts were tested for their *in vitro* inhibitory effects on 5-lipoxygenase (LO) and COX (Sendl *et al*., 1992). Thiosulfinates from *A. tuberosum* Rottl. were reported to inhibit cell proliferation and induced apoptosis via the caspase-dependent and -independent pathways in human prostate cancer PC-3 cells (Kim *et al*., 2008).

Ajoene, was reported to demonstrate NSAID-like drug properties when it inhibits prostaglandin synthesis in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages *in vitro*. This novel finding may be an important understanding for future exploration of the chemoprevention for gastrointestinal carcinomas (Dirsch and Vollmar, 2001). It was also demonstrated that ajoene had anti-proliferation and apoptosis inducing properties when tested against the human myeloid leukaemic cells (Dirsch *et al*., 2002; Li *et al*., 2002).

*S*-allylcysteine (SAC) decreased toxicity levels of doxorubicin in the heart and liver of mice (Mostafa *et al*., 2000). Sundaresan and Subramanian (2003) showed that SAC exerted tumour inhibitory effects in their study by enhancing the antioxidant levels to prevent formation of free radicals and hindering in N-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis in rats. In a recent study, SAC was found to reduce cell adhesion and invasion of the human breast tumour MDA-MB-231 cells (Gapter *et al*., 2008).
Shukla et al., (2002) had observed significant cytotoxicity of DAS in a dose-dependent manner against Ehrlich-ascites tumour cells. The potential of a few types of allyl sulfides (e.g. diallysulfide, diallyldisulfide and diallyltrisulfide) in inducing phase II detoxification enzymes were defined by Fukao et al. (2004). In another study, DADS was found to induce cell cycle arrest at G2/M phase and apoptosis in a time- and dose-dependent manner in human lung cancer A549 cells (Wu et al., 2005).
CHAPTER 3
MATERIALS AND METHODS

3.1 Selection and Extraction of Plant Material

3.1.1 Plant Materials

The seven edible *Allium* plants investigated in the present study are *A. cepa* L. (onion), *A. fistulosum* L. (spring onion), *A. porrum* L. (leek), *A. sativum* L. (garlic), *A. ursinum* L. (ramson) and two variants of *A. tuberosum* Rottl., namely ‘bunga kuchai’ and ‘daun kuchai’ (Plate 3.1). These *Alliums* were selected for this study because they were the most commonly consumed *Alliums* and have various medicinal values.

Two kg of each plant were purchased from the local market and bulbs used in the experiment should not be spoiled or infected in any way. As for the leafy types, it is important to choose healthy bunches and leaves that are yellow or torn or damaged in any way should be removed. This is important to ward off any possibilities of the imperfections affecting the plant metabolism and thus cause the formation of unwanted products that could alter the plant’s phytochemical constituent’s activity (Houghton and Raman, 1998).

3.1.2 Drying of Plant Materials

The scaly skin layers was removed from the bulbs of *A. cepa* L., *A. sativum* L. and *A. ursinum* L. and these bulbs were cut into thin slices to allow faster dehydration. As for the other *Alliums*, the leaves were cut into shorter pieces to allow easy dehydration. The pieces of the *Alliums* were dried in the oven (Memmert) at 50°C. The dried samples
Plate 3.1 The *Allium* spp. selected from the Malaysian local market for investigation
were then ground into powdered form. The drying of these plant materials is crucial in order to avoid the presence of water in the extracts produced (Houghton and Raman, 1998).

### 3.1.3 Infusion Method

The infusion method was used in the present study to screen for different bioactivities of the samples because this method does not involve heating and hence help preserve most of the phytochemicals in the samples. In this method, 100.0 g of the powdered *Allium* spp. was infused with petroleum ether ([40°C-60°C], Surechem) for 72 hours at room temperature (27°C) in a conical flask. The mixture was subjected to intermittent shaking in an incubator shaker (New Brunswick Scientific CO. Inc.) to ensure optimum exposure of the samples to the solvent. After that, the mixture was filtered and the sample was allowed to air-dry in between changes of extraction solvent to avoid carry-over of previous solvent used (Houghton and Raman, 1998). The infusion process was carried on using chloroform ([stabilized 0.60–1.00% ethanol], Systerm) and then methanol (Systerm).

The obtained extracts were then subjected to evaporation using the rotary evaporator (Buchi Co.) to remove excess solvent. The concentrated extracts were weighed and then kept in airtight specimen bottles that were stored in a refrigerator (0–4°C) to prevent the loss of material. The process was repeated until all the *Allium* spp. powders were extracted. The yield for each *Allium* spp. was calculated using the formula provided:
Crude extracts obtained from the samples were used for cytotoxic activity screening, determination of antioxidant activity and their chemical contents and anti-HPV type 16 E6 oncoprotein studies using ICC techniques.

3.2 Stock Preparation

3.2.1 Storage of Plant Extracts

20.0 mg of each *Allium* spp. crude extract was diluted in 1.0 ml dimethyl sulfoxide (DMSO, Sigma) and stored at -20°C until use.

3.3 Preparation of Cell Culture Materials and Solutions

3.3.1 Sterilization of Glassware and Apparatus

Washing

Glassware such as pipettes, beakers and bottles were first soaked in 7X detergent (Flowlab) for 24 hours before submerging them into tap water for another 24 hours. All glassware were rinsed with distilled water and dried in the oven (Memmert).
**Autoclaving**

Pipettes were removed from the oven (Memmert) and the end openings of the pipettes were stuffed with cotton wool. The pipettes were placed in pipette canisters and heat-sterilized in the oven at 180°C for two hours.

**Dry Sterilization**

Other glassware was sterilized using the autoclave (P-Selecta) at 121°C for 15 to 20 minutes.

**3.3.2 Preparation of Cell Culture Medium and Solutions**

**Basic RPMI 1640**

A bottle of powdered RPMI 1640 with L-glutamine, earl salts and without sodium bicarbonate (RPMI 1640, Sigma) was dissolved in one litre of distilled water. 2.0 g of sodium bicarbonate (NaHCO₃, Merck) and 0.52 g of N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid (HEPES, Sigma) were added and mixed well. The media was filter-sterilized using the 0.20 μm filter membrane (Schleicher & Schuell). The media was kept at 4°C and the stability of the media prepared is two months.

**10% Supplemented RPMI 1640**

This medium was prepared using the following materials and mixed well in a beaker: 90.0 ml of sterile basic RPMI 1640 medium, 10.0 ml of 10% foetus bovine serum (FBS, Flowlab), 2.0 ml of penicillin/streptomycin (PAA Lab) and 1.0 ml of Amphostat B (Flowlab). The media was filter-sterilized into a bottle using the 0.2 μm filter membrane (Schleicher & Schuell) and kept at 4°C.
20% Supplemented RPMI 1640

This supplemented RPMI 1640 medium was made up of 45.0 ml of 10% supplemented RPMI 1640 medium and 5.0 ml of FBS (Flowlab). The medium was filter-sterilized using 0.2 µm filter membrane (Schleicher & Schuell) into a bottle and kept at 4°C.

Freezing Medium

The freezing medium was prepared using 50% of FBS (Flowlab), 40% of basic RPMI 1640 medium (Sigma) and 10% of DMSO (Sigma). The medium was mixed well and filter-sterilized using a 0.2 µm filter membrane (Schleicher & Schuell) into a bottle and kept at 4°C. It was recommended to use freshly prepared media for cryopreservation of cells.

FBS

Untreated FBS (– 20°C) was thawed using a water bath (Grant) at 56°C for 30 minutes to inactivate its complements. It was then stored at -20°C.

0.25% Trypsin-EDTA

0.25% Trypsin-EDTA was prepared by dissolving 0.25 g of powdered trypsin (Amresco) and 0.03 g of ethylenediaminetetraacetic acid (EDTA, Sigma) in 100 ml of sterile PBS. The solution was filter-sterilize using a 0.2 µm filter membrane (Schleicher & Schuell). It was kept at -20°C.
Phosphate-buffered Saline (PBS) pH 7.2

The PBS consisted of 1.52 g of sodium phosphate anhydrous (Na$_2$HPO$_4$, BDH-Analar), 0.58 g potassium dihydrogen orthophosphate (KH$_2$PO$_4$, Merck) and 8.5 g sodium chloride (NaCl, BDH-Analar), which were dissolved in one litre of distilled water. The solution was adjusted to a pH of 7.2 and it was filtered using a 0.2 μm filter membrane (Schleicher & Schuell) into a sterile bottle before autoclaving. Sterile PBS was kept at room temperature.

3.4 Cell Culture

3.4.1 Cell Lines

The normal human lung cell line (MRC-5) (ATCC Lot Number 3929229), the human epidermal carcinoma of cervix HPV type-16 contained-cell line (CaSki) (ATCC Lot Number 3327067) and human colon carcinoma cell line (HCT-116) (ATCC Lot Number 3540494) were used in this study. The cells were obtained from the American Type Culture Collection (ATCC, USA). The Trypan Blue exclusion dye method was used to test the viability of the cells and these frozen cells were stored in a -196°C liquid nitrogen tank prior to use.

3.4.2 Reviving of Cell Lines

A provial of MRC-5 cells was removed from the liquid nitrogen storage tank and placed into a beaker of ice. For quick thawing, it was transferred into a water bath (Grant) maintained at 37°C. The cells were then transferred into a polypropylene centrifuge tube (Falcon) containing with 1.0 ml of 20.0% supplemented EMEM medium (Sigma). The tube was centrifuged using a centrifuge machine (Clements 2000) at 1,000 rpm for
five minutes before discarding the supernatant. The pellet was resuspended in 7.0 ml of
20.0% supplemented EMEM medium in a tissue culture flask (Nunc). The flask was
then incubated in a 5.0% carbon dioxide (CO₂) water-jacket incubator (Shel Lab) at
37°C. The same procedures were applied for CaSki cells using RPMI 1640 medium
(Sigma) and HCT-116 cells using McCoy’s medium (Sigma).

3.4.3 Maintenance of Cell Cultures

MRC-5 cells were revived and cultured in 10.0% supplemented EMEM medium in the
tissue culture flask (Nunc) and incubated at 37°C in 5.0% CO₂ water-jacket incubator
(Shel Lab). The 10.0% EMEM supplemented medium contained 10.0% FBS (PAA
Lab). Cells were examined daily using the inverted microscope (Olympus) for signs of
bacterial or fungal contamination. The supplemented medium in the flask was replaced
with fresh 10.0% supplemented EMEM medium twice a week or when a colour change
was observed. Cultured cells were sub-cultivated when the cell concentration exceeds
2.0 X 10⁶ per ml. Then the flasks were further incubated. The CaSki cells were
maintained using RPMI 1640 medium while HCT-116 cells were maintained using
McCoy’s medium.

3.4.4 Sub-cultivation of Cells

The medium in the tissue culture flasks (Nunc) was discharged when the MRC-5 cells
attached themselves onto the surface of the flasks. The flasks were rinsed using 10.0 ml
of PBS (pH 7.2). Then, 3.0 ml of PBS and 1.0 ml 0.25% trypsin-EDTA were added into
the tissue culture flasks so that the cells would detach themselves from the surface of
the flasks. The cells were incubated at 37°C for five minutes in 5.0% CO₂ water-jacket
incubator (Shel Lab) before centrifugation at 1,000 rpm for five minutes with 1.0 ml of 10.0% supplemented EMEM medium.

Supernatant in the polypropylene centrifuge tube (Falcon) was discarded and the pellet of MRC-5 cells was resuspended in 3.0 ml 10.0% supplemented EMEM medium. 1.0 ml of the MRC-5 cell suspension was transferred into each of the tissue culture flasks containing 6.0 ml of 10.0% supplemented EMEM medium. The flasks were incubated in 5.0% CO$_2$ water-jacket incubator (Shel Lab) at 37°C. CaSki cells were sub-cultivated using the same procedures with RPMI 1640 medium while HCT-116 cells with McCoy’s medium.

3.4.5 Cryopreservation of Cells

MRC-5 cells with confluent or sub-confluent monolayer were fed with fresh 10% supplemented EMEM medium 24 hours before freezing. After that, the MRC-5 cells were treated as described in section 3.4.4. After centrifugation, the supernatant was discarded and the pellet was resuspended in 3.0 ml of freezing medium. 1.0 ml of MRC-5 cell suspension was transferred into each of the 3 provial. The provials were then left in ice in a polystyrene box and freeze at -70°C for at least four hours or overnight before transferring into a liquid nitrogen tank and stored at -196°C until use. The same cryopreservation procedures were used for CaSki and HCT-116 cells.
3.5 Cytotoxicity Screening

3.5.1 Serial Dilution of Plant Extracts

Plant extracts with the concentrations of 1.0, 10.0, 50.0 and 100.0 µg/ml were prepared from a 20.0 mg/ml crude extract stock using 10.0% DMSO and kept in -20°C prior to use.

3.5.2 Preparation of Cytotoxic Screening Materials and Solutions

0.4% Tryphan Blue

0.4% Tryphan Blue was prepared by dissolving 0.2 g of powdered Tryphan Blue (Sigma) into 50.0 ml of distilled water. It was kept at room temperature prior to use.

10% DMSO

To produce a 10% DMSO stock solution, DMSO (Sigma) was mixed with distilled water in a ration of 1:9. It was recommended to use freshly prepared DMSO stock for cytotoxicity testing.

NR Stock Solution

0.04 g of powdered NR (ICN Biomedicals Inc.) was dissolved in 10.0 ml of distilled water to obtain a final concentration NR stock of 4 mg/ml. The stock was wrapped with aluminium foil and kept at 4°C prior to use.

NR Medium

The NR medium was prepared using 10% supplemented RPMI 1640 media to obtain a final concentration of 50 µg/ml medium in a centrifuge tube (Falcon). The solution was
wrapped in aluminium foil and kept in the dark at room temperature. When using, the solution was centrifuged twice for ten minutes at 1,500 rpm to remove any precipitate of dye crystals.

**Washing Solution**

To prepare the washing solution, 1.0 ml of formaldehyde (Sigma) and 1.0 g of calcium chloride (CaCl₂, Sigma) was dissolved in 100.0 ml distilled water. The solution was kept at 4°C prior to use.

**Resorb Solution**

The resorb solution was prepared with 1.0 ml of glacial acetic acid (BDH-Analar), 50.0 ml of absolute ethanol (Sigma) and 50.0 ml of distilled water. It was mixed well and stored at 4°C prior to use.

**3.5.3 Cell Enumeration**

1.0 ml of 0.25% of trypsin was added into a tissue culture flask containing confluent MRC-5 cells and incubated for five minutes in the 5.0% CO₂ water-jacket incubator (Shel Lab) at 37°C. Then the detached cells were centrifuged at 1,000 rpm for five minutes with 1.0 ml of 10.0% supplemented EMEM medium. Supernatant in the polypropylene centrifuge tube (Falcon) was discarded and the pellet of MRC-5 cells was resuspended in 1.0 ml 10.0% supplemented EMEM medium.

100.0 µl of the MRC-5 cell suspension was aliquoted into a provial containing 900.0 µl of 0.4% Tryphan Blue and mixed well. The density of the viable cells was counted in a
haemacytometer (Scherf) by loading 20.0 µl of the MRC-5 cell suspension with dye at the edges of the cover slip covering the haemacytometer. The MRC-5 cell suspension with dye will flow into the counting chambers by capillary action. Following that, the haemacytometer was observed under an inverted microscope (Olympus) and the living cells would appear clearly and were counted. A concentration of $5 \times 10^4$ MRC-5 cells per ml of cell suspension was prepared following the formula below:

\[
P_1 \times 10^5 \times V_1 = P_2 \times V_2
\]

where $P_1$ : average number of viable cells counted using heamacytometer

$P_2$ : the desired cell concentration of cell suspension

$V_1$ : volume of cell suspension required

$V_2$ : volume of 10% supplemented medium needed for seeding

$10^5$ : counting chamber conversion and dilution factor with dye

The same procedures were applied to CaSki and HCT-116 cells except a concentration of $3 \times 10^4$ cells per ml of cell suspension was used for CaSki cells for cell enumeration.

### 3.5.4 Incubation of Cells with Plant Extract

190.0 µl of MRC-5 cells was seeded into treatment wells of a 96-well plate (Nunc) while 200.0 µl of MRC-5 cells were required for the non-treatment wells. The 96-well plate was then incubated in the 5.0% CO$_2$ water-jacket incubator (Shel Lab) at 37°C for 24 hours. The MRC-5 cells were allowed to adhere and achieve 70-80.0% confluence.
before the addition of plant extracts. After 24 hours, different volumes of 1.0, 10.0, 50.0 and 100.0 μg/ml of each crude extract were then added to the respective wells. The 96-well plates were then further incubated for 72 hours in the 5.0% CO₂ water-jacket incubator at 37°C. The assay was carried out in triplicates. The well with untreated MRC-5 cells served as negative control while MRC-5 cells treated with doxorubicin (Sigma) acts as a positive control. The same procedures were applied to CaSki and HCT-116 cells.

3.5.5 NR Assay

The NR assay was performed according to the method of Borenfreund and Puerner (1984) with slight modifications. Cell lines were incubated in multiple doses of sample (1.0, 10.0, 50.0 and 100.0 μg/ml) to determine the effective concentration of growth inhibition. At the end of the 72 hours incubation period, the 96-well plates were inverted to remove the content of the wells which was then replaced with 200.0 μl medium containing 50.0 μg/ml NR. The plates were incubated for another three hours to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. After incubation period, the media were removed and cells were washed quickly with a fixative (washing solution).

The dye was eluted from the cells by adding 200.0 μl of resorb solution and further incubated for another 30 minutes at room temperature with rapid agitation on a benchtop incubator/shaker (LT Biomax 500). Dye absorbance was measured at 540 nm using ELISA reader (Titertek Multiskan® MCC/340). Three replicate plates were used
to determine the cytotoxicity of each extract. The percentage of inhibition was determined using the formula below:

\[
\text{Percentage of Inhibition (\%)} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ control}} \times 100\%
\]

where A : absorbance reading

The cytotoxic activity was expressed as the IC\(_{50}\) value estimated from the graphical interpolation of the dose-response curve, which is defined as the concentration of extract that causes 50% of inhibition or cell death (Chiang et al., 2003; Chen et al., 1988; Geran et al., 1972). According to National Cancer Institute guidelines, plant extracts exhibiting IC\(_{50}\) value of 20.0 µg/ml or less is considered active in cytotoxicity testing (Chen et al., 1988; Geran et al., 1972). The 50% cell growth inhibition concentration is considered the best indicator of cytotoxicity as it is taken from the middle of the dose-response curve (Jover et al., 1992).

3.6 Antioxidant Activity Screening

3.6.1 Preparation of Antioxidant Assay Stocks and Solutions

0.2 mg/ml β-carotene Stock

0.0002 g of β-carotene (BASF, Lucarotin 30A) was diluted in 1.0 ml of chloroform (Sigma) to produce a 0.2 mg/ml β-carotene stock. It was kept in a test tube wrapped in aluminium foil at 4°C until use.
2.51% (v/v) Linoleic Acid

This solution was prepared by mixing 2.64 ml of linoleic acid (~95.0%) (Sigma) with 97.36 ml of absolute ethanol (~99.8%, Systerm) to produce a 100.0 ml of 2.51% (v/v) linoleic acid solution.

0.2M Phosphate Buffer (pH 6.6)

(A) To prepare 0.2M monobasic sodium phosphate (NaH$_2$PO$_4$, Merck) buffer, 27.80 g of NaH$_2$PO$_4$ was dissolved in one litre of distilled water.

(B) To prepare 0.2M dibasic sodium phosphate (Na$_2$HPO$_4$.7H$_2$O, BDH-Analar) buffer, 53.65 g of Na$_2$HPO$_4$.7H$_2$O was dissolved in one litre of distilled water.

To prepare 0.2M phosphate buffer (pH 6.6), 62.5 ml of buffer (A) was mixed with 37.5 ml of buffer (B) and 200.0 ml of distilled water. The pH was adjusted to 6.6 and kept at room temperature until use.

0.05M Phosphate Buffer (pH 7.0)

To obtain a 0.05M phosphate buffer, 39.0 ml of buffer (A) above was added to 61.0 ml of buffer (B) of above with 200.0 ml of distilled water to obtain a 0.2M phosphate buffer. The pH of the buffer was then adjusted to 7.0. To get a 0.05M phosphate buffer, 100.0 ml of the 0.2M phosphate buffer (pH 7.0) was diluted gradually with 300.0 ml of distilled water. The pH was monitored so that it would remain as pH 7.0.
30.0% (w/v) Ammonium Thiocyanate

30.0 g of ammonium thiocyanate (NH₄SCN, Sigma) was dissolved in 100.0 ml of distilled water and mixed well. The solution was kept at room temperature in the dark until use.

3.5% (v/v) Hydrochloric Acid

9.46 ml of hydrochloric acid [-37%] (HCl, BDH-Analar) was diluted with 100.0 ml of distilled water to obtain a 3.5% (v/v) HCl solution. The solution was kept at room temperature prior to use.

0.02M Ferrous Chloride in 3.5% (v/v) Hydrochloric Acid

0.2535 g of ferrous chloride (FeCl₂, Sigma) was dissolve in 3.5% (v/v) hydrochloric acid to give a 0.02M FeCl₂ solution. It was kept wrapped in aluminium foil at room temperature and in the dark until use.

Ascorbic Acid Stock Solution

A 400.0 µg/ml ascorbic acid (Sigma) was prepared in methanol (Systerm) and kept in a bottle wrapped with aluminium foil at 4°C until use.

DPPH Stock Solution

A stock solution of 8.0 mg/ml of DPPH was prepared using methanol (Systerm). The solution was kept at 4°C in a bottle wrapped with aluminium foil.
1.0% (w/v) Potassium Ferricyanide

This solution was prepared by dissolving 0.1 g potassium ferricyanide (K₃[Fe(CN)₆], Systerm) in 10.0 ml of distilled water. The solution was wrapped with aluminium foil and kept in the dark at room temperature prior to use.

10.0% (w/v) Trichloroacetic Acid

10.0% (w/v) trichloroacetic acid (TCA, Merck) solution was prepared with 10.0 g of trichloroacetic acid and 100.0 ml of distilled water. The solution was mixed well and kept at room temperature until use.

0.1% (w/v) Ferric Chloride

0.01 g of ferric chloride (FeCl₃, Systerm) was dissolved with 10.0 ml of distilled water to give a 0.1% (w/v) ferric chloride solution. The solution was wrapped with aluminium foil and kept in the dark at room temperature prior to use.

Gallic Acid Stock Solution

0.5 g of gallic acid (Sigma) was dissolved in 10.0 ml of absolute ethanol (~ 99.8%, Systerm) and diluted to volume with distilled water in a 100.0 ml volumetric flask. The stock was kept in a bottle at 4°C prior to use.

Sodium Carbonate Solution

200.0 g of anhydrous sodium carbonate (Na₂CO₃, Sigma) was dissolved in 800.0 ml of distilled water. The solution was bring to a boil and cooled before adding a few sodium carbonate crystals. The solution was left for 24 hours before filtering. The filtered
solution was topped-up with distilled water to one litre in volume. The solution was kept at room temperature in a bottle.

2.0% (w/v) AlCl₃

2.0 g of AlCl₃ (Systerm) was diluted in 100.0 ml of distilled water and mixed well. The solution was kept in the dark at room temperature until use.

3.6.2 Antioxidant Bioassay Methods

β–carotene Bleaching Inhibition Assay

The antioxidant activity of the plant extracts was determined using the β-carotene bleaching inhibition assay (Cheung et al., 2003). 0.2 mg/ml β-carotene (BASF, Lucarotin 30A) stock in chloroform (Sigma) was prepared and kept in a test tube wrapped in aluminium foil in the dark until use. The plant extracts were prepared fresh with methanol (Systerm) in concentrations of 4.0, 8.0, 16.0 and 20.0 mg/ml and kept aside until use. Meanwhile, a mixture containing 1.0 ml of β-carotene stock, 20.0 µl of linoleic acid (Sigma) and 200.0 µl of Tween 80 (Sigma) was prepared in a round bottom flask. After the chloroform in this mixture was removed using the rotary evaporator (Buchi), 50.0 ml of oxygenized sterile distilled water was immediately added.

After vigorous stirring, a liposome solution was obtained and 5.0 ml of this emulsion was added into every test tube containing 0.2 ml of the prepared plant extracts in different concentrations of 4.0, 8.0, 16.0 and 20.0 mg/ml respectively. These test tubes were placed in a water bath (Grant) of 50°C for 120 minutes and the absorbance value
for each test tube was read at 470 nm at intervals of 20 minutes, beginning from \( t=0 \) minute until the end of the experiment, \( t=120 \) minutes. Control was prepared using the emulsion and 0.2 ml of solvent (petroleum ether, chloroform and methanol respectively) while blank contained all the earlier chemicals except \( \beta \)-carotene. All assays were done in triplicates. Butylated hydroxyanisole (BHA, Sigma) was used as standard positive reference. The antioxidant activity was calculated using the formula below:

\[
\frac{\ln (a/b)}{t} \quad \text{where } \ln \text{ : natural log;}
R = \frac{a}{t} \quad a : \text{absorbance reading of sample at } t = 0 \text{ min;}
\]

\[
b : \text{absorbance reading of sample at } t = t \text{ min}
\]

When the \( R \) value is obtained, the antioxidant activity (%) is calculated using:

\[
\text{Antioxidant Activity (\%) = } \frac{(R \text{ control} - R \text{ sample})}{R \text{ control}} \times 100\%.
\]

The FTC Assay

The FTC assay was performed based on the method of Kikuzaki and Nakatani (1993) with slight modification. 4.0 mg of crude extract from each sample was mixed with 4.0 ml of absolute ethanol (~ 99.8%, Systerm), 4.1 ml of 2.51% (v/v) linoleic acid (Sigma) in absolute ethanol (~ 99.8%), 8.0 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of distilled water in different screw-capped vials and incubated in an oven at 40°C in the dark.

The extent of oxidation was measured every 24 hours by mixing 0.1 ml of the mixture solution from the vials with 9.7 ml of 75.0% ethanol. After that, 0.1 ml of 30.0% (w/v) \( \text{NH}_4\text{SCN} \) was added followed by 0.1 ml of 0.02M \( \text{FeCl}_2 \) in 3.5% (v/v) hydrochloric acid
and mixed well. The absorbance reading was taken after three minutes at 494 nm. Absorbance reading was taken every 24 hours until one day after the absorbance of the negative control has reached its maximum. Negative control consists of reaction mixture in which crude extract was substituted with methanol itself. On the day the negative control gave its maximum absorbance reading, lipid peroxidation was taken as 100% (Emami et al., 2007). BHA (Sigma) was used as standard positive reference. The antioxidant activity was measured using the formula below (Emami et al., 2007):

\[
\text{Antioxidant Activity (\%)} = \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \times 100
\]

where \( A \) : absorbance reading

The lower the absorbance value, the greater the antioxidant activity of the crude extracts.

**The DPPH Free Radical Scavenging Assay**

The DPPH free radical scavenging assay was carried out with slight modifications according to Yamaguchi et al. (1998). In this method, the plant extracts were pre-screened using a simplified protocol before a more comprehensive DPPH assay can be carried out. The reaction mixtures for pre-screening were prepared following the details in Table 3.1. The reaction mixtures were allowed to react for 30 minutes at room temperature in the dark. After 30 minutes, the absorbance value of each reaction mixture was read at 520 nm using a UV spectrophotometer (Hitachi U2000) and
recorded. Methanol (Systerm) was used as blank. The pre-screening was done in triplicates and ascorbic acid (Sigma) and BHA (Sigma) were used as standard positive references. Reaction mixture without crude extract was replaced with methanol (Systerm) to serve as a negative control.

Table 3.1 The pre-screening protocol of *Allium* spp. crude extracts in DPPH scavenging activity assay

<table>
<thead>
<tr>
<th>Concentration of Extract (mg/ml)</th>
<th>Methanol (µl)</th>
<th>Crude Extract (µl)</th>
<th>DPPH Solution (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test</strong></td>
<td>5.0</td>
<td>725.0</td>
<td>250.0</td>
</tr>
<tr>
<td><strong>Negative Control</strong></td>
<td>-</td>
<td>975.0</td>
<td>-</td>
</tr>
</tbody>
</table>

The plant extracts which performed a 50.0% or more inhibition were further tested using a more detailed protocol as tabulated in Table 3.2 using the same procedures above and the IC₅₀ value was determined.

Table 3.2 The determination of IC₅₀ value of *Allium* spp. crude extracts in DPPH scavenging activity assay

<table>
<thead>
<tr>
<th>Concentration of Extract (mg/ml)</th>
<th>Methanol (µl)</th>
<th>Crude Extract (µl)</th>
<th>DPPH Solution (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>725.0</td>
<td>250.0</td>
<td>25.0</td>
</tr>
<tr>
<td>4.0</td>
<td>775.0</td>
<td>200.0</td>
<td>25.0</td>
</tr>
<tr>
<td>3.0</td>
<td>825.0</td>
<td>150.0</td>
<td>25.0</td>
</tr>
<tr>
<td>2.0</td>
<td>875.0</td>
<td>100.0</td>
<td>25.0</td>
</tr>
<tr>
<td>1.0</td>
<td>925.0</td>
<td>50.0</td>
<td>25.0</td>
</tr>
<tr>
<td><strong>Negative Control</strong></td>
<td>-</td>
<td>-</td>
<td>25.0</td>
</tr>
</tbody>
</table>
The IC$_{50}$ value is defined as the concentration that causes a decrease in the initial amount of DPPH radicals by 50.0% (Huang et al., 2005) as can be calculated using the formula below:

\[
\text{Percentage of Inhibition (\%)} = \frac{(A \text{ control} - A \text{ sample})}{A \text{ control}} \times 100\% 
\]

where A : absorbance reading

The Reducing Power Assay

The reducing power assay was carried out according to Oyaizu (1986) with some slight modifications. The crude extracts were prepared in concentrations of 5.0, 10.0, 15.0 and 20.0 mg/ml in methanol (Systerm). In separate test tubes, these extracts were added with 2.5 ml of 0.2M phosphate buffer (pH 6.6) and mixed well. Then 2.5 ml of 1.0% (w/v) K$_3$[Fe(CN)$_6$] solution was added into the respective test tubes and mixed well. The reaction mixture was then allowed to incubate in a water bath (Grant) at 50°C for 20 minutes before adding in 2.5 ml of 10.0% (w/v) TCA solution. The reaction mixture was centrifuged at 1000 rpm for ten minutes.

Following this, 2.5 ml of the upper supernatant layer was extracted and added with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) FeCl$_3$ solution and then mixed well. The absorbance reading of the reaction mixture was taken at 700 nm. All tests were run in triplicates and averaged. Ascorbic acid (Sigma) was used as positive standard reference. The higher the absorbance value, the greater the reducing power of the crude extracts.
3.6.3 Determination of Total Phenolic Content

The total phenolic content of the selected Alliums was determined using the Folin-Ciocalteau reagent (BDH-AnalaR) with slight modifications (Cheung et al., 2003; Singleton and Rossi, 1965). A calibration curve with effective range of phenol concentrations of 0.0, 50.0, 100.0, 150.0, 250.0 and 500.0 mg/L was prepared by adding 0.0, 1.0, 2.0, 3.0, 5.0 and 10.0 ml of the gallic acid stock into a 100.0 ml volumetric flask and diluted to volume with distilled water (Appendix A).

To determine the total phenolic content, 20.0 µl of either calibration solution, crude extracts (20.0 mg/ml) prepared in methanol (Systerm) was added into different test tubes containing 1.58 ml of distilled water. Following that, 100.0 µl of the Folin-Ciocalteau reagent (BDH-AnalaR) were added and the solution was mixed well. The reactive mixture was left for 30 seconds to eight minutes before adding 300.0 µl of sodium carbonate solution and the solution was then mixed well using the vortex mixer.

The reactive mixture was left to incubate at 40°C in the dark for 30 minutes. The absorbance of each mixture was read at 765 nm using a spectrophotometer (Hitachi U2000). Methanol was used as blank. The determination of total phenolic content was carried out in triplicates and the mean of three absorbance readings was used and the total phenolic content was expressed in mg gallic acid equivalents (GAEs)/100.0 g sample.
3.6.4 Determination of Total Flavonoid Content

The estimation of total flavonoid content of the selected *Allium* was done using the AlCl₃ colorimetric assay with slight modifications (Meda *et al.*, 2005; Arvouet-Grand *et al.*, 1994). A calibration curve with effective range of concentrations of 0.0, 5.0, 10.0, 15.0, 25.0 and 50.0 mg/L was prepared by adding 0.0, 0.01, 0.02, 0.03, 0.05 and 0.1 ml of the quercetin (Sigma) stock (5.0 mg/ml) into a 10.0 ml volumetric flask and diluted to volume with methanol (Systerm) (Appendix A).

Briefly, 5.0 ml of extract (5.0 mg/ml) was added to the same amount of 2.0% AlCl₃ in methanol and mixed well. The reactive mixture was left for ten minutes at room temperature before reading the absorbance at 414 nm. A blank sample was prepared by substituting the crude extract with methanol. All tests were run in triplicates and the absorbance readings were averaged and expressed as mg quercetin equivalents (QEs)/100.0 g sample.

3.7 Anti-HPV 16 E6 Protein Testing

The anti-HPV 16 E6 protein activity of *Allium* crude extracts against CaSki cells was tested according to Nurhayati (1997) with slight modifications.

3.7.1 Serial Dilution of Plant Extracts

0.18 ml from the each sample’s stock was diluted in 2.82 ml of sterile distilled water to produce a stock concentration of 1200 µg/ml. This stock was then further diluted into final concentrations of 600.0 µg/ml, 450.0 µg/ml, 300.0 µg/ml, 150.0 µg/ml, 75.0 µg/ml, 37.5 µg/ml and 3.0 µg/ml respectively. All diluted stocks were kept at -20°C
until use.

### 3.7.2 Incubation of Cells with Plant Extracts

2.0 ml of cultured CaSki cells (3 X 10⁴ cells per ml of suspension) in a tissue flask was added with 1.0 ml of a serial diluted stock sample (e.g. plant extract yield by petroleum ether) to give a total concentration of 200.0 μg/ml in a grand volume of 3.0 ml. Then, the same procedure was applied to prepare several other tissue flasks containing the same amount of cells (2.0 ml) to give various total concentrations of 150.0 μg/ml, 100.0 μg/ml, 50.0 μg/ml, 25.0 μg/ml, 12.5 μg/ml, and 1.0 μg/ml respectively for the same sample used. The cells were incubated at 37°C in the 5.0% CO₂ incubator for three days before based onto welled Teflon-coated glass slides.

### 3.7.3 Fixation of Cells onto Slides

Cultured CaSki cells that were incubated with plant extracts were transferred into a tissue culture tube and centrifuged at 1,000 rpm for ten minutes. After the supernatant was decanted, the cells were washed twice in PBS (pH 7.2). Cells were then resuspended in fresh PBS. Using a Pasteur pipette, one drop of cell suspension was carefully placed onto each well on the welled Teflon-coated glass slides. The slides were then placed into sterile Petri dishes and left for overnight incubation in the 5.0% CO₂ incubator. When the cells were confluent, slides were removed and washed twice in PBS (pH 7.6). The CaSki cells were fixed using acetone for eight minutes.

### 3.7.4 Detection of HPV E6 Protein

The HPV E6 proteins in this study were detected using the ICC technique as described
in section 3.8. ICC staining was done using the three-step indirect Labelled Strepavidin-Biotin (LSAB) Peroxidase Kit (Dako) and the DAB Substrate System (Dako).

3.8 ICC

3.8.1 Preparation of Solutions

Various Concentrations of Ethanol

Ethanol solutions of 80%, 90% and 95% were prepared by diluting absolute ethanol (~99.8%, Systerm) with distilled water into the concentration needed.

PBS pH 7.6

This PBS consisted of 1.5 g of potassium phosphate anhydrous (K$_2$HPO$_4$, BDH-Analar), 0.2 g KH$_2$PO$_4$ (Merck) and 7.75 g NaCl (BDH-Analar), which were dissolved in one litre of distilled water. The solution was adjusted to a pH of 7.6 and kept at room temperature.

3% (v/v) H$_2$O$_2$

3 ml of 30% H$_2$O$_2$ (BDH-Analar) was diluted with 7 ml of distilled water to obtain a preparation of 3% H$_2$O$_2$. It was kept at room temperature.

Antibody Dilution

The anti-HPV type 16 E6 protein monoclonal antibody (Chemicon) was diluted using steriled PBS (pH 7.6) with the ratio of 1:50 and kept in a provial prior to use at 4°C.
3.8.2 Rehydration

The cells were rehydrated in decreasing concentrations of ethanol; 100.0%, 95.0%, 90.0% and 80.0% for two minutes each and then washed in PBS (pH 7.6) for five minutes on the rocker.

3.8.3 Elimination of Endogenous Peroxidase Activity

Each well was then incubated with 40.0 µl of 3.0% (v/v) H₂O₂ for ten minutes at 37°C to remove the endogenous peroxidase activity. The slides were rinsed in PBS (pH 7.6) for five minutes.

3.8.4 ICC Staining

The areas surrounding each well were blot dry before filling each appropriate well with 40.0 µl of anti-HPV 16 E6 monoclonal antibody (Chemicon). The slides were then incubated for 90 minutes at 37°C. Following this, the slides were washed twice in PBS (pH 7.6) for 15 minutes each before adding secondary antibodies (Labelled Strepavidin-Biotin® 2 system) into the respective wells. Incubation was allowed to proceed for ten minutes at 37°C.

The slides were then washed again in PBS for five minutes before incubation with 40.0 µl of strepavidin-HRP conjugate for ten minutes at 37°C. Next, the slides were washed in PBS for another five minutes. The slides were then filled with 40.0 µl of DAB and incubated at 37°C. Incubation was stopped when the desired colour intensity developed (approximately five to 20 minutes).
The slides were then rinsed in distilled water before counterstaining with Mayers Hematoxylin for two minutes. Slides were rinsed again with distilled water before being mounted with pre-warmed glycergel (53°C). The slides were left to dry in the dark for 24 hours before analyzing them under the light microscope (Leica Microsystems, USA). Microscopic images (40X) were taken for comparison purposes to determine the expression levels of E6 oncoprotein after treatment with crude extracts.
4.1 Extraction of Selected *Allium* spp.

Seven *Allium* plants were selected for extraction purposes. These plants were dried and grounded before subjected to infusion. The yield from each of the plant samples after drying and grounded are shown in Table 4.1.

**Table 4.1 The dry weight (g) yield of the *Allium* samples**

<table>
<thead>
<tr>
<th>Plant Samples (Wet Weight of 2 kg)</th>
<th>Dry Weight (Grounded) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1130.0</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>532.0</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>608.0</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>1290.0</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>1210.0</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>579.0</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>501.0</td>
</tr>
</tbody>
</table>

The seven *Allium* plants were extracted using the infusion method with petroleum ether, chloroform and methanol. The percentages of yield calculated for all 21 crude extracts are tabulated in Table 4.2. *A. cepa* L. gave the highest total crude extract yield of 10.56% while *A. ursinum* L. produced the lowest amount of total crude extract yield with 1.15%. Generally for all samples, the total percentage yields of crude methanol extracts (ranged 1.05% to 7.55%) were higher than the petroleum ether (ranged 0.06% to 0.68%) and chloroform extracts (ranged 0.04% to 2.33%).
Table 4.2 The percentage yield of crude extracts of selected *Allium* spp.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Solvent Used</th>
<th>Weight of Crude Extract (g)</th>
<th>Yield of Crude Extract (%)</th>
<th>Total Yield of Crude Extract (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. cepa</em> L.</td>
<td>Petroleum ether</td>
<td>0.8701</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>1.0682</td>
<td>0.09</td>
<td>5.32</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>58.2346</td>
<td>5.15</td>
<td></td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>Petroleum ether</td>
<td>1.0867</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>1.7194</td>
<td>0.32</td>
<td>3.62</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>16.4678</td>
<td>3.10</td>
<td></td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>Petroleum ether</td>
<td>2.0646</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>6.1428</td>
<td>1.01</td>
<td>6.97</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>34.1907</td>
<td>5.62</td>
<td></td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>Petroleum ether</td>
<td>1.3788</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>1.6359</td>
<td>0.13</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>14.1941</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>Petroleum ether</td>
<td>0.7013</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>0.4675</td>
<td>0.04</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>12.6684</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>Petroleum ether</td>
<td>2.4889</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>8.8091</td>
<td>1.52</td>
<td>8.13</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>35.8095</td>
<td>6.18</td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>Petroleum ether</td>
<td>3.3918</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>11.6816</td>
<td>2.33</td>
<td>10.56</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>37.8280</td>
<td>7.55</td>
<td></td>
</tr>
</tbody>
</table>
4.2 Cytotoxicity Screening of Selected *Allium* spp.

The *Alliums* in the present study were screened for their cytotoxic activity using the rapid, inexpensive and convenient method of NR assay. The various crude extracts from the *Alliums* were tested against normal human lung cell line (MRC-5), human epidermal carcinoma of cervix HPV type-16 contained-cell line (CaSki) and human colon carcinoma cell line (HCT-116). MRC-5 cells were used as buffer cells in the NR assay because they maintain most of the basic biochemical distinctiveness of undifferentiated cells when compared to its CaSki and HCT-116 counterparts.

An initial cytotoxicity screening was done using doxorubicin as the positive control against the three cell lines. Doxorubicin is from the anthracycline family which originated from mutant strains of *Streptomyces peucetius* var. *caesius* and was originally described as anti-tumour antibiotics (Rubin and Hait, 2003). This drug interacts with the DNA template and inhibits RNA synthesis, which consequently blocks the transcription process (Montgomery, 1981). The growth inhibition activity and IC\(_{50}\) value of doxorubicin against MRC-5, CaSki and HCT-116 cells are shown in Table 4.3 and Table 4.4, respectively.

**Table 4.3 The percentage of inhibition of doxorubicin against MRC-5, CaSki and HCT-116 cell lines**

<table>
<thead>
<tr>
<th>Concentration of Doxorubicin (μg/ml)</th>
<th><em>Percentage of Inhibition</em> (%) ± SD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRC-5</td>
<td>CaSki</td>
</tr>
<tr>
<td>0.00001</td>
<td>8.01 ± 0.75</td>
<td>5.75 ± 3.69</td>
</tr>
<tr>
<td>0.001</td>
<td>9.83 ± 0.86</td>
<td>15.30 ± 3.67</td>
</tr>
<tr>
<td>0.01</td>
<td>11.52 ± 0.88</td>
<td>24.13 ± 1.42</td>
</tr>
<tr>
<td>0.1</td>
<td>12.05 ± 2.22</td>
<td>70.20 ± 1.32</td>
</tr>
</tbody>
</table>
Table 4.3 continued

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>21.03 ± 2.71</td>
<td>76.49 ± 0.44</td>
<td>37.21 ± 5.46</td>
</tr>
<tr>
<td>1.0</td>
<td>51.97 ± 1.24</td>
<td>84.73 ± 0.37</td>
<td>72.11 ± 4.48</td>
</tr>
<tr>
<td>10.0</td>
<td>73.76 ± 1.01</td>
<td>87.86 ± 0.34</td>
<td>80.39 ± 3.68</td>
</tr>
<tr>
<td>100.0</td>
<td>78.10 ± 0.63</td>
<td>89.25 ± 0.79</td>
<td>84.59 ± 1.61</td>
</tr>
</tbody>
</table>

* Percentage of inhibition as mean of triplicate experiments ± standard deviation (SD)

Table 4.4 IC<sub>50</sub> value of doxorubicin against MRC-5, CaSki and HCT-116 cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC-5</td>
<td>1.000</td>
</tr>
<tr>
<td>CaSki</td>
<td>0.006</td>
</tr>
<tr>
<td>HCT-116</td>
<td>0.360</td>
</tr>
</tbody>
</table>

In the present study, it was obvious that doxorubicin is a cytotoxic agent that is more effective against CaSki cells than HCT-116 cells. It was the least cytotoxic towards MRC-5 cells.

4.2.1 *Allium* spp. against MRC-5

The crude petroleum ether, chloroform and methanol extracts of the selected *Alliums* were tested against the MRC-5 cell line and their dose-response curves are presented in Figure 4.1(a-c). All of the plant extracts were not active against MRC-5.

Crude Petroleum Ether Extracts

As shown in Figure 4.1(a), at the highest concentration of petroleum ether extract (i.e. 100 µg/ml), *A. tuberosum* Rottl. (daun kuchai) exerted the strongest cell growth
inhibitory effects of 40.04%±0.90 while \textit{A. sativum} L. exhibited the poorest inhibitory effect of 10.00%±0.46 against the MRC-5 cell line.

### Crude Chloroform Extracts

When tested at concentration 100 µg/ml extract, the chloroform extracts showed less than 50% of growth inhibition rates against MRC-5 cells. The chloroform extract of \textit{A. tuberosum} L. (daun kuchai) showed highest growth inhibition of 37.65%±2.10 towards the MRC-5 cells while \textit{A. tuberosum} L. (bunga kuchai) exhibited the lowest inhibitory effect of 14.09%±3.80 (Figure 4.1(b)).

### Crude Methanol Extracts

The cytotoxic activities of the selected methanol \textit{Allium} spp. extracts are illustrated in Figure 4.1(c). Methanol extract of \textit{A. cepa} L. demonstrated highest MRC-5 growth inhibition of 43.35%±1.28 at 100 µg/ml extract. The weakest cytotoxic activity was exhibited by \textit{A. tuberosum} Rottl. (bunga kuchai) with 7.17%±0.97 at the same concentration of extract.
Figure 4.1(a) Growth inhibition of MRC-5 cells by crude petroleum ether extracts of selected *Allium* spp. using the NR assay
Figure 4.1(b) Growth inhibition of MRC-5 cells by crude chloroform extracts of selected *Allium* spp. using the NR assay

*IC*$_{50}$ $> 100.0$ µg/ml
Figure 4.1(c) Growth inhibition of MRC-5 cells by crude methanol extracts of selected *Allium* spp. using the NR assay.
4.2.2 *Allium* spp. against CaSki

The dose-response curves of the cytotoxic activities of the crude petroleum ether, chloroform and methanol extracts of the selected *Allium* against CaSki cells are presented in Figure 4.2(a-c). All the plants’ extracts were not active against CaSki cells.

In general, CaSki cells were seen to be most sensitive towards *A. sativum* L. among other plants because at concentration 100 µg/ml, all three extracts of *A. sativum* L. exhibited similarly high inhibitory effects against CaSki cells. At the same concentration of extract, CaSki cells were least sensitive towards petroleum ether extract of *A. tuberosum* Rottl. (daun kuchai) and both chloroform and methanol extracts of *A. ursinum* L. as indicated by their low cell killing percentage.

**Crude Petroleum Ether Extracts**

Figure 4.2(a) illustrates the dose-response curves of the various *Allium* spp. petroleum ether extracts against CaSki cells. At the concentration of 100 µg/ml extract, *A. cepa* L. exhibited the highest inhibition rate on CaSki cells’ growth with 65.49%±3.26 while *A. tuberosum* Rottl. (daun kuchai) exhibited weak cell growth inhibition of 17.86%±5.63.

Based on the results, three petroleum ether extracts were observed to exhibit more than 50% cell growth inhibition towards the CaSki cells in the present study. *Allium cepa* L., *A. sativum* L. and *A. ursinum* L. had each caused a 50% CaSki cells’ growth inhibition by registering IC$_{50}$ values of 83.0 µg/ml, 88.0 µg/ml and 98.5 µg/ml, respectively. However, these extracts were considered not active (Chen *et al.*, 1988; Geran *et al.*, 1972). Other petroleum ether extracts did not register an IC$_{50}$ value.
Crude Chloroform Extracts

The dose-response curves of the chloroform *Allium* spp. extracts is shown in Figure 4.2(b). Overall, the chloroform extracts were not active towards CaSki cells and did not register a 50% growth inhibition rate. Chloroform extract of *A. sativum* L. was seen to inhibit CaSki cells’ growth with the highest inhibition rate of 48.95%±12.00. The lowest inhibition rate was exhibited by *A. ursinum* L. with 23.33%±11.77.

Crude Methanol Extracts

The methanol *Allium* extracts were not active towards CaSki cells when tested using the NR assay and no IC\(_{50}\) value was registered. The methanol extract of *A. sativum* L. was observed to exert the highest growth inhibition rate of 47.58%±8.77 at the concentration of 100 µg/ml extract. The poorest inhibition was demonstrated by *A. ursinum* L. with 23.75%±4.22 (Figure 4.2(c)).

4.2.3 *Allium* spp. against HCT-116

The growth inhibitions of HCT-116 cells by crude petroleum ether, chloroform and methanol extracts are shown in Figures 4.3(a-c). All petroleum ether extracts were not active against HCT-116 cells and none gave 50% of growth inhibition activity. In the present study, HCT-116 cells were most sensitive towards the extracts of *A. porrum* L. because all three extracts of *A. porrum* L. exerted highest inhibitory effects against HCT-116 cells at the highest concentration of 100 µg/ml extract.
Figure 4.2(a) Growth inhibition of CaSki cells by crude petroleum ether extracts of selected *Allium* spp. using the NR assay
Figure 4.2(b) Growth inhibition of CaSki cells by crude chloroform extracts of selected *Allium* spp. using the NR assay
Figure 4.2(c) Growth inhibition of CaSki cells by crude methanol extracts of selected Allium spp. using the NR assay
**Crude Petroleum Ether Extracts**

The dose-response curves of the petroleum ether extracts of *Allium* spp. against HCT-116 cells are illustrated in Figure 4.3(a). It was observed that at the 100 µg/ml, the petroleum ether extract of *A. tuberosum* Rottl. (bunga kuchai) demonstrated highest inhibition rate of 38.76±1.29% while *A. fistulosum* L. exerted lowest inhibition rate of 19.84±2.47.

**Crude Chloroform Extracts**

Based on the results, the growth inhibition activity of HCT-116 cells exhibited by chloroform *A. porrum* L. extract was highest with 63.29±3.29%. At concentration 100 µg/ml, this extract had inhibited more than 50% HCT-116 cells’ growth and registered an IC₅₀ value of 38 µg/ml. On the other hand, *A. cepa* L. chloroform extract was the least effective in inhibiting cell growth by exerting a weak 17.69±7.00 activity (Figure 4.3(b)).

**Crude Methanol Extracts**

Figure 4.3(c) shows the growth inhibitions of HCT-116 cells by the methanol extracts of the *Alliums* spp. in the present study. At the concentration of 100 µg/ml extract, *A. porrum* L. exhibited most effective growth inhibition activity of 44.28±6.87. Treatment with 100 µg/ml *A. fistulosum* L. extracts poorly inhibited HCT-116 cells’ growth with 26.72±3.62 of inhibitory activity.
Figure 4.3(a) Growth inhibition of HCT-116 cells by crude petroleum ether extracts of selected *Allium* spp. using the NR assay

*IC$_{50}$* > 100.0 µg/ml
Figure 4.3(b) Growth inhibition of HCT-116 cells by crude chloroform extracts of selected *Allium* spp. using the NR assay
Figure 4.3(c) Growth inhibition of HCT-116 cells by crude methanol extracts of selected *Allium* spp. using the NR assay
4.2.4 Comparison of Cytotoxic Activities of Selected *Allium* spp. against MRC-5, CaSki and HCT-116

In the present study, the crude extracts of *Allium* spp. had each demonstrated different inhibitory activities against the three MRC-5, CaSki and HCT-116 cell lines. The ranges of cell killing activities varied amongst the crude extracts and are presented in Table 4.5(a-c).

**Crude Petroleum Ether Extracts**

Generally, the results revealed that the petroleum ether extracts inhibited the proliferation of MRC-5, CaSki and HCT-116 cells in a dose-dependent manner and the petroleum ether extracts were not active against the three cell lines. At the concentration 100 μl/ml, the highest killing percentage was exhibited by *A. cepa* L. against CaSki cells with 65.49%±3.26 while the least effective inhibitor against MRC-5 cells was petroleum ether *A. sativum* L. extract with 10.00%±0.46. The cell killing percentages range at various concentrations of petroleum ether extracts against MRC-5, CaSki and HCT-116 cells are presented in Table 4.5(a).

**Table 4.5(a) The cell killing percentage of *Allium* spp. at various concentrations of crude petroleum ether extracts**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>1.0 μg/ml</th>
<th>10.0 μg/ml</th>
<th>50.0 μg/ml</th>
<th>100.0 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC-5</td>
<td>7.19 - 28.04</td>
<td>7.72 - 29.51</td>
<td>7.93 - 35.59</td>
<td>10.00 - 40.04</td>
</tr>
<tr>
<td>CaSki</td>
<td>1.29 - 17.41</td>
<td>6.21 - 24.73</td>
<td>16.64 - 42.02</td>
<td>17.86 - 65.49</td>
</tr>
<tr>
<td>HCT-116</td>
<td>0.81 - 15.63</td>
<td>6.21 - 24.38</td>
<td>15.21 - 33.97</td>
<td>19.84 - 38.76</td>
</tr>
</tbody>
</table>
Crude Chloroform Extracts

Likewise, chloroform extracts of *Allium* spp. also demonstrated cell proliferation inhibitory activity against MRC-5, CaSki and HCT-116 cells in a dose-dependent manner. Overall, the chloroform extracts were not active against the three cell lines. At 100 μg/ml, the chloroform extract of *A. porrum* L. exhibited 63.29%±3.29 inhibitory effect against HCT-116 cells’ growth while *A. tuberosum* Rottl. (bunga kuchai) recorded the lowest killing percentage of 14.09%±3.80 against MRC-5 cells.

Although *A. porrum* L. had caused a 50% cell growth inhibition rate against HCT-116 cells at the concentration 38 μg/ml extract but this crude extract was considered not active (Chen *et al.*, 1988; Geran *et al.*, 1972). The cell killing percentages range at various concentrations of chloroform extracts against MRC-5, CaSki and HCT-116 cells are presented in Table 4.5(b).

### Table 4.5(b) The cell killing percentage of *Allium* spp. at various concentrations of crude chloroform extracts

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>1.0 μg/ml</th>
<th>10.0 μg/ml</th>
<th>50.0 μg/ml</th>
<th>100.0 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT-116</td>
<td>0.79 - 26.71</td>
<td>3.02 - 32.81</td>
<td>9.26 - 57.29</td>
<td>17.69 - 63.29</td>
</tr>
</tbody>
</table>

Crude Methanol Extracts

The cell killing percentages range at various concentrations of methanol *Allium* extracts against MRC-5, CaSki and HCT-116 cells are presented in Table 4.5(c). Based on the
results, the methanol extracts of *Allium* spp. were observed to exhibit cell proliferation inhibition in a dose-dependent manner. The methanol extracts were found to be not active against the three cell lines and no IC$_{50}$ was registered. At 100 μg/ml, *A. sativum* L. had exhibited the highest inhibition against CaSki cells with 47.58%±8.77 while *A. tuberosum* Rottl. (bunga kuchai) was the least effective inhibitor against MRC-5 cells with 7.17%±0.97.

**Table 4.5(c) The cell killing percentage of Allium spp. at various concentrations of crude methanol extracts**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Killing Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 μg/ml</td>
</tr>
<tr>
<td>MRC-5</td>
<td>2.30 - 25.90</td>
</tr>
<tr>
<td>CaSki</td>
<td>2.62 - 24.08</td>
</tr>
<tr>
<td>HCT-116</td>
<td>1.94 - 24.40</td>
</tr>
</tbody>
</table>

Generally, it was observed that CaSki cells were most sensitive towards the activities of the various *Allium* spp. petroleum ether extracts and HCT-116 was most sensitive to chloroform *Allium* spp. extracts. Treatment with 100 μg/ml extracts had caused 47.58 to 65.49% and 38.76 to 63.29% killing percentages of CaSki and HCT-116 cells respectively.

In the present study, MRC-5 cells were consistently sensitive to all crude extracts of the *Allium* plants in the present study. At concentration of 100 μg/ml extract, the cell growth inhibition activities of petroleum ether, chloroform and methanol extracts were
similarly against MRC-5 and ranged from 37.65 to 43.35%. The IC_{50} values of the *Allium* crude extracts in the present study were summarized and presented in Table 4.6.

**Table 4.6 The IC_{50} values of *Allium* spp. crude extracts tested against MRC-5, CaSki and HCT-116 cell lines**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Crude Extract</th>
<th>IC_{50} Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MRC-5</td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>Petroleum ether</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>&gt; 100.0</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>&gt; 100.0</td>
</tr>
</tbody>
</table>
4.3 Antioxidant Activities of Selected *Allium* spp.

Extracts of the selected *Allium* spp. were evaluated for antioxidant activities using the β-carotene bleaching inhibition assay, the FTC assay, the DPPH assay and the reducing power assay. In the present study, antioxidant activities of the *Allium* crude extracts were categorized according to Vimala and Ilham (1999). Antioxidant activity was considered as high if the activity was ≥ 70%, moderate if activity was ≥ 50% and low if activity was < 50%. The assays were performed using whole extracts of the *Allium* plants because an individual bioactive compound may exhibit better biological properties in the presence of other compounds in the extract, and therefore be more beneficial than isolated constituents (Barros *et al*., 2008).

4.3.1 The β-Carotene Bleaching Inhibition Assay

The inhibition of oxidative destruction of β-carotene by *Allium* crude extracts were evaluated in this β-carotene bleaching inhibition assay. BHA was used as standard positive reference and its inhibition activity is tabulated in Table 4.7.

<table>
<thead>
<tr>
<th>Concentration of BHA (mg/ml)</th>
<th>4.0</th>
<th>8.0</th>
<th>16.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant Activity* (%)</td>
<td>44.76 ± 5.52</td>
<td>56.55 ± 11.96</td>
<td>73.48 ± 8.54</td>
<td>77.87 ± 7.76</td>
</tr>
</tbody>
</table>

* Antioxidant activity as a mean of triplicate experiments ± standard deviation (SD)
Overall results indicated that the crude petroleum ether extracts were most effective in the inhibition of β-carotene bleaching when compared to its crude chloroform and methanol counterparts. The resulting antioxidant activities by crude petroleum ether, chloroform and methanol extracts of the selected *Allium* plants are shown in Figure 4.4(a), (b) and (c), respectively.

**Crude Petroleum Ether Extracts**

The antioxidant activity of petroleum ether extracts range from 11.25% to 91.10% for concentrations of 4 to 20 mg/ml (Figure 4.4(a)). Overall results showed that at all concentrations of extract tested, petroleum ether extracts of *A. cepa* L. and *A. porrum* L. exhibited antioxidant activities better than BHA while *A. ursinum* L. petroleum ether extract was poorest in inhibiting lipid peroxidation (LPO).

At the lowest concentration of 4 mg/ml, most of the *Allium* extracts were better than BHA in inhibiting LPO. The petroleum ether extracts of *A. cepa* L., *A. porrum* L. and both variants of *A. tuberosum* Rottl. were more effective than BHA (i.e. 44.76% ± 5.52) in inhibiting the oxidative destruction of β-carotene even at the lowest concentration tested (i.e. 4 mg/ml). At the same concentration, the highest inhibition activity was exhibited by *A. tuberosum* Rottl. (daun kuchai) petroleum ether extract (i.e. 82.03%±3.02) while the lowest antioxidant activity was exhibited by *A. ursinum* L. with 11.25%±1.25.

At 8 mg/ml, all the *Allium* exhibited β-carotene bleaching inhibition activity better than BHA except for *A. ursinum* L. which exhibited poorest β-carotene bleaching inhibition
activity of 11.39%±1.03. The highest β-carotene bleaching inhibition activity was exhibited by *A. tuberosum* Rottl. (daun kuchai) with 91.61%±2.61 at the same concentration of extract. At the highest concentration of extract tested (i.e.20 mg/ml), only two *Alliums* exhibited antioxidant activity better than BHA. The petroleum ether extracts of *A. cepa* L. and *A. porrum* L. exhibited antioxidant activities of 91.10%±3.71 and 86.03%±9.97 respectively while *A. ursinum* L. reported weakest β-carotene bleaching inhibition activity of 25.20%±2.51.

**Crude Chloroform Extracts**

The antioxidant activity exhibited by chloroform extracts of *Allium* plants in the present study ranged from 26.68% to 90.77% at concentrations of 4 to 20 mg/ml of extracts (Figure 4.4(b)). Generally it was observed that at all concentrations of extract, chloroform extracts of *A. porrum* L. and *A. ursinum* L. were more effective than BHA in inhibiting the bleaching of β-carotene. The antioxidant activity of chloroform of *A. cepa* L. extract was poorest at all concentrations of extract tested.

All chloroform extracts but *A. cepa* L. were better than BHA in inhibiting LPO at the lowest concentration of 4 mg/ml. At the same concentration of extract, the highest antioxidant activity was shown by *A. ursinum* L. chloroform extract with 83.97%±2.00 while *A. cepa* L. chloroform extract gave weakest inhibitory effects of 26.68%±4.81. At the highest concentration of extract tested (i.e. 20 mg/ml), chloroform extract of *A. ursinum* L. exhibited the highest β-carotene bleaching inhibition activity of 90.77%±2.01 while the poorest antioxidant activity was exerted by *A. cepa* L. with 34.18%±4.01.
Crude Methanol Extracts

The methanol extracts of the selected *Allium* in the present study were the least potential in inhibiting β-carotene bleaching. As illustrated in Figure 4.4(c), the antioxidative activities of *Allium* plants’ methanol extracts were recorded in the range of 14.92% to 77.37% at concentrations of 4 to 20 mg/ml of extracts. It was observed that all methanol *Allium* extracts except *A. sativum* L. were less effective than BHA in inhibiting β-carotene bleaching.

At 4 mg/ml, only methanol extract of *A. sativum* L. exerted antioxidant activity better than BHA while the rest of the methanol extracts performed weaker antioxidant activity compared to that by BHA. At this concentration, the methanol extract of *A. sativum* L. exhibited antioxidant activity of 54.67%±1.18. However, at the highest concentration of 20 mg/ml, the methanol extract of *A. sativum* L. exhibited antioxidant activity of 77.37%±2.51 which was as good as that of BHA (i.e. 77.87%±7.76).

Overall weakest antioxidant activity was exerted by methanol extract of *A. fistulosum* L. at 4, 16 and 20 mg/ml (i.e.14.92%±0.90, 29.89%±0.26 and 26.48%±0.89 respectively) while *A. ursinum* L. methanol extract exhibited poorest antioxidant activity of 24.77%±1.77 at 8 mg/ml.
Figure 4.4(a) Antioxidative activity of crude petroleum ether extracts from selected *Allium* spp. in the β-carotene bleaching inhibition assay
Figure 4.4(b) Antioxidative activity of crude chloroform extracts from selected *Allium* spp. in the β-carotene bleaching inhibition assay
Figure 4.4(c) Antioxidative activity of crude methanol extracts from selected *Allium* spp. in the β-carotene bleaching inhibition assay.
4.3.2 The FTC Assay

In the present study, the inhibition of initial formation of lipid peroxides by selected *Allium* plants was determined using the FTC assay. By inhibiting linoleic acid oxidation, the formation of lipid peroxides can be hindered. The inhibition activities reflect the antioxidant activities of the *Allium* samples (Figure 4.5(a-c)). In this study, BHA was used as standard positive reference and its antioxidant activity is presented in Table 4.8.

In this assay, it was observed that absorbance values of the negative control increased until day eight, and decreased the day after. Hence, the results for this assay were based on the readings obtained until day eight. The results showed that crude chloroform extracts of the selected *Allium* spp. were the most active in inhibiting initial formation of lipid peroxides followed by petroleum ether extracts and then methanol extracts.

Table 4.8 The antioxidant activity of BHA (0.2 mg/ml) as determined by the FTC assay

<table>
<thead>
<tr>
<th>Incubation Day(s)</th>
<th>Antioxidant Activity* of BHA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>36.70 ± 0.14</td>
</tr>
<tr>
<td>Day 2</td>
<td>52.34 ± 1.51</td>
</tr>
<tr>
<td>Day 3</td>
<td>64.90 ± 2.24</td>
</tr>
<tr>
<td>Day 4</td>
<td>74.95 ± 0.24</td>
</tr>
<tr>
<td>Day 5</td>
<td>79.77 ± 2.38</td>
</tr>
<tr>
<td>Day 6</td>
<td>82.69 ± 0.32</td>
</tr>
<tr>
<td>Day 7</td>
<td>83.74 ± 0.41</td>
</tr>
<tr>
<td>Day 8</td>
<td>84.55 ± 0.08</td>
</tr>
</tbody>
</table>

* Antioxidant activity as mean of triplicate experiments ± standard deviation (SD)
Crude Petroleum Ether Extracts

In general, it was observed that the antioxidant activities of the *Allium* petroleum ether extracts increased with the increasing days of incubation. Only petroleum ether extract of *A. fistulosum* L. exhibited antioxidant activity better than BHA for seven incubation days and was as good as BHA on the eighth day of incubation. The petroleum ether extracts of *A. sativum* L. exhibited the poorest antioxidant activities throughout the eight days incubation period and this extract reached its maximum antioxidant potential as soon as the third day of incubation while other *Allium* extracts only reached their maximum antioxidant activity from the fifth day of incubation onwards.

On the first day of incubation, petroleum ether extracts of *A. fistulosum* L., *A. porrum* L. and *A. tuberosum* Rottl. (bunga kuchai) were more effective in inhibiting the formation of lipid peroxides than BHA by exhibiting antioxidant activities of 40.35%±2.25, 37.47%±3.91 and 42.28%±1.00, respectively. The petroleum ether extracts of *A. cepa* L., *A. porrum* L., *A. ursinum* L. and *A. tuberosum* Rottl. (bunga kuchai) reached their maximum antioxidant activity on day five of incubation.

On the eighth day of the experiment, the petroleum ether extracts of *A. fistulosum* L. and *A. tuberosum* Rottl. (daun kuchai) were the most effective extracts and were as good as BHA. These extracts exhibited antioxidant activity of 83.33%±0.11 and 83.70%±0.34, respectively. On the same day, petroleum ether *A. sativum* L. extract exerted weakest inhibition activity of 5.99%±0.28 (Figure 4.5(a)).
**Crude Chloroform Extracts**

In the main, the antioxidant activities of *Alliums* were observed to increase with the increasing days of incubation (Figure 4.5(b)). In the present study, the chloroform extracts of *A. fistulosum* L. and two *A. tuberosum* Rottl. variants (i.e. bunga kuchai and daun kuchai) exhibited antioxidant activities higher than BHA throughout the eight days of experiment while chloroform *A. sativum* L. extract was evidently seen with lowest antioxidant activity in the eight days of incubation.

Likewise its petroleum ether counterpart, chloroform extract of *A. sativum* L. reached its maximum antioxidant activity on day three of incubation. Chloroform extract of *A. cepa* L. reached its maximum antioxidant activity (i.e. 71.78%±3.26) on day five while *A. ursinum* L. chloroform extract exhibited its highest antioxidant activity (i.e. 75.74%±0.29) on day six of incubation. The rest of the chloroform *Allium* extracts were exhibiting high antioxidant activity (≥ 70% activity) on the day eight.

On day one, most of the chloroform *Allium* extracts exerted antioxidant activity lower than BHA. However, chloroform extracts of *A. fistulosum* L. and both variants of *A. tuberosum* Rottl. were more effective in inhibiting the formation of lipid peroxides than BHA. The chloroform extract of *A. fistulosum* L. exerted antioxidant activity of 44.18%±3.31 while *bunga kuchai* and *daun kuchai* exhibited lipid peroxidation inhibition activity of 51.46%±3.06 and 40.34%±1.09, respectively.

Based on the results on the eighth day of the experiment, five of the chloroform *Allium* extracts studied showed high potential (≥ 70% activity) in retarding the oxidation of
linoleic acid in the present study. Chloroform extracts of *A. fistulosum* L., *A. porrum* L. and both variant of *A. tuberosum* Rottl. antioxidant activities’ were most convincing in inhibiting the formation of the red coloured ferric thiocyanate complex as these samples exerted inhibition properties that were better than BHA (i.e. 84.55%±0.08). The poorest antioxidant activity was exhibited by chloroform extract of *A. sativum* L. with 15.30%±0.40.

**Crude Methanol Extracts**

In general, all methanol extracts demonstrated antioxidant activities lower than BHA throughout the eight days of experiment. Most of the methanol *Allium* extracts exhibited antioxidant activity of less than 50% in inhibiting autoxidation of linoleic acid (Figure 4.5(c)). Overall, the methanol extract of *A. ursinum* L. was the most effective in inhibiting the formation of FTC in the present study.

The methanol extracts of *A. fistulosum* L., *A. porrum* L. and *A. sativum* L. reached their peak antioxidant activity on day four while *A. ursinum* L. and *A. tuberosum* Rottl. (bunga kuchai) exhibited maximum FTC inhibition activity on day five. On the eighth day, the methanol extract of *A. tuberosum* Rottl. (daun kuchai) reached its maximum antioxidant activity of 36.96%±0.34 while the weakest anti-FTC formation activity was exerted by methanol *A. fistulosum* L. extract with 4.02%±0.37.
Figure 4.5(a) The inhibition of lipid oxidation by crude petroleum ether extracts of *Allium* spp. (0.2 mg/ml) as determined by the FTC assay
Figure 4.5(b) The inhibition of lipid oxidation by crude chloroform extracts of *Allium* spp. (0.2 mg/ml) as determined by the FTC assay.
Figure 4.5(c) The inhibition of lipid oxidation by crude methanol extracts of *Allium* spp. (0.2 mg/ml) as determined by the FTC assay
4.3.3 The DPPH Radical Scavenging Activity Assay

The free radical scavenging activities of the crude *Allium* extracts were evaluated using the DPPH radical in this assay. The crude petroleum ether, chloroform and methanol extracts of selected *Allium* spp. was first pre-screened for their DPPH radical scavenging activity. These extracts were tested for their scavenging activities at the concentration of 5 mg/ml. Extract(s) that produced 50 or more percentage of inhibition of the DPPH radical were regarded as positive extracts and were selected for further evaluation of its DPPH radical scavenging activity using various concentrations (1, 2, 3, 4 and 5 mg/ml).

The percentages of inhibition obtained were used to plot a dose-response curve from which IC$_{50}$ values were extrapolated. IC$_{50}$ value is the concentration at which the crude extract exhibits a 50% inhibition activity against the DPPH radical (Huang *et al.*, 2005). In general, the lower the IC$_{50}$ value, the higher the radical scavenging/antioxidant power. Ascorbic acid and BHA were used as standard positive references and their IC$_{50}$ values were determined as 12.5 μg/ml (0.0125 mg/ml) and 16.8 μg/ml (0.0168 mg/ml), respectively (Figure 4.6).

When all *Allium* crude extracts were pre-screened at the concentration of 5 mg/ml, three extracts produced 50% of inhibition or above (i.e. positive extracts). They were the petroleum ether extract of *A. tuberosum* Rottl. (daun kuchai) and chloroform extracts of *A. cepa* L. and *A. ursinum* L.. The petroleum ether extract of *A. tuberosum* Rottl. (daun kuchai) exhibited 51.23%±0.00 of DPPH scavenging effects while the chloroform *A.
cepa L. and A. ursinum L. extracts gave 86.27±0.05 and 72.45±0.00 of inhibition respectively.

**Determination of DPPH Radical Scavenging Activity of Selected Allium spp.**

The three positive extracts namely petroleum ether extract of *A. tuberosum* Rottl. (daun kuchai) and chloroform extracts of *A. cepa* L. and *A. ursinum* L. were retested at various concentrations of 1, 2, 3, 4 and 5 mg/ml. Based on their results, a dose-response curve was plotted to determine their IC$_{50}$ values. It was observed that the DPPH radical scavenging activities of these crude extracts increased with the increment of concentrations of extract.

Overall results of DPPH radical inhibition by the three positive extracts were simplified and presented in Table 4.9. The determination of IC$_{50}$ values for the active extracts of *A. cepa* L., *A. ursinum* L. and *A. tuberosum* Rottl. (daun kuchai) are illustrated in Figure 4.7. The IC$_{50}$ values obtained from the active extracts of *A. cepa* L., *A. ursinum* L. and *A. tuberosum* L. (daun kuchai) are summarized and compared with ascorbic acid and BHA in Table 4.10.
Table 4.9 The anti-DPPH radical activities of crude petroleum ether extracts of *A. tuberosum* Rottl. (daun kuchai) and crude chloroform extracts of *A. cepa* L. and *A. ursinum* L.

<table>
<thead>
<tr>
<th>Concentration of Active Crude Extract (mg/ml)</th>
<th>Percentage of Inhibition* (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>4.07 ± 2.46</td>
<td>19.16 ± 3.16</td>
</tr>
<tr>
<td>2.0</td>
<td>7.20 ± 5.89</td>
<td>22.20 ± 1.24</td>
</tr>
<tr>
<td>3.0</td>
<td>24.82 ± 4.92</td>
<td>26.87 ± 3.13</td>
</tr>
<tr>
<td>4.0</td>
<td>53.33 ± 1.36</td>
<td>44.00 ± 4.66</td>
</tr>
<tr>
<td>5.0</td>
<td>58.18 ± 2.69</td>
<td>73.68 ± 1.25</td>
</tr>
</tbody>
</table>

* Percentage of inhibition as DPPH scavenging activities as a mean of triplicate experiments ± standard deviation (SD)

Table 4.10 Summary of IC₅₀ values of the active crude extracts and positive references, ascorbic acid and BHA in the DPPH radical scavenging activity assay

<table>
<thead>
<tr>
<th>Active Extracts</th>
<th>IC₅₀ Value (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai) Crude Petroleum Ether Extract</td>
<td>3.89</td>
</tr>
<tr>
<td><em>A. cepa</em> L. Crude Chloroform Extract</td>
<td>4.20</td>
</tr>
<tr>
<td><em>A. ursinum</em> L. Crude Chloroform Extract</td>
<td>4.55</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>0.0125</td>
</tr>
<tr>
<td>BHA</td>
<td>0.0168</td>
</tr>
</tbody>
</table>
Ascorbic Acid - IC<sub>50</sub> = 12.5 µg/ml
BHA - IC<sub>50</sub> = 16.8 µg/ml

Figure 4.6 The determination of IC<sub>50</sub> values of ascorbic acid and BHA as standard positive references in the DPPH radical scavenging activity assay
Figure 4.7 The determination of IC$_{50}$ values of the active crude extracts of *A. cepa* L., *A. ursinum* L. and *A. tuberosum* Rottl. (daun kuchai) in the DPPH radical scavenging activity assay

- *A. cepa* L. - IC$_{50}$ = 4.20 mg/ml
- *A. ursinum* L. - IC$_{50}$ = 4.55 mg/ml
- *A. tuberosum* Rottl. (Daun kuchai) - IC$_{50}$ = 3.89 mg/ml

--- Shows the extrapolation of IC$_{50}$ value from the dose-response curve of extract
4.3.4 The Reducing Power Assay

The reducing capability was measured by the transformation of Fe$^{3+}$ to Fe$^{2+}$ in the presence of *Allium* crude extracts at 700 nm. The reducing capacity of an extract may serve as an indicator of its potential antioxidant activity. Higher absorbance of the reaction mixture indicates greater reductive potential. Ascorbic acid was used as standard positive reference in this assay and its reducing powers are tabled in Table 4.11.

<table>
<thead>
<tr>
<th>Concentration of Ascorbic Acid (mg/ml)</th>
<th>5.0</th>
<th>10.0</th>
<th>15.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance Reading*</td>
<td>2.780A±0.017</td>
<td>3.004A±0.019</td>
<td>3.067A±0.030</td>
<td>3.089A±0.025</td>
</tr>
</tbody>
</table>

*Absorbance reading as a mean of triplicate experiments ± standard deviation (SD)*

In the present study, all crude extracts of *Allium* spp. demonstrated reducing power in a dose-dependent manner (Figure 4.8(a-c)) and the summary of their reducing powers is tabled in Table 4.12(a-c). The *Allium* plant’s reducing power increased with the increasing concentration of plant extracts used.

**Crude Petroleum Ether Extracts**

As illustrated in Figure 4.8(a), all petroleum ether extracts exhibited low reducing power at all concentrations of extracts (5, 10, 15 and 20 mg/ml) when compared to that by ascorbic acid. The inset in Figure 4.8(a) showed that the reducing powers of
petroleum ether extracts of *Allium* plants ranged from 0.014A to 0.529A at concentrations of 5 to 20 mg/ml. At all concentrations of extract, petroleum ether extract of *A. porrum* L. was the most active reductant.

At 5 mg/ml, the reducing power of petroleum ether extract of *A. porrum* L. was with 0.475A±0.00 and then 0.500A±0.01 at 10 mg/ml, increased to 0.521±0.01 at 15 mg/ml and 0.529A±0.01 at 20 mg/ml. On the other hand, the petroleum ether extract of *A. tuberosum* Rottl. (bunga kuchai) was the least reductive. At the lowest concentration (i.e. 5 mg/ml), the reducing power of *bunga kuchai* was 0.019A±0.005, increased to 0.035A±0.004 at 10 mg/ml, 0.055A±0.006 at 15 mg/ml and then at 20 mg/ml, its reducing power was 0.063A±0.003.

At the highest concentration of 20 mg/ml, the reducing power of petroleum ether *A. cepa* L., *A. fistulosum* L., *A. sativum* L. and *A. tuberosum* Rottl. (daun kuchai) were 0.309A±0.004, 0.100A±0.002, 0.070A±0.002 and 0.402A±0.002 respectively. The reducing powers of the selected *Allium* petroleum ether extracts are shown in Table 4.12(a).

**Table 4.12(a) The reducing power of various concentrations of crude petroleum ether extracts of selected *Allium* spp.**

<table>
<thead>
<tr>
<th>Concentration of Extract (mg/ml)</th>
<th>Absorbance Reading*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>0.112 ± 0.005</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>0.017 ± 0.001</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.475 ± 0.002</td>
</tr>
</tbody>
</table>
Table 4.12(a) continued

<table>
<thead>
<tr>
<th></th>
<th>Absorbance (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. sativum L.</td>
<td>0.023 ± 0.002</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>0.014 ± 0.003</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (bunga kuchai)</td>
<td>0.019 ± 0.005</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (daun kuchai)</td>
<td>0.175 ± 0.006</td>
</tr>
<tr>
<td><strong>10.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td>A. cepa L.</td>
<td>0.164 ± 0.002</td>
</tr>
<tr>
<td>A. fistulosum L.</td>
<td>0.056 ± 0.002</td>
</tr>
<tr>
<td>A. porrum L.</td>
<td>0.500 ± 0.006</td>
</tr>
<tr>
<td>A. sativum L.</td>
<td>0.036 ± 0.002</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>0.036 ± 0.007</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (bunga kuchai)</td>
<td>0.035 ± 0.004</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (daun kuchai)</td>
<td>0.208 ± 0.002</td>
</tr>
<tr>
<td><strong>15.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td>A. cepa L.</td>
<td>0.235 ± 0.004</td>
</tr>
<tr>
<td>A. fistulosum L.</td>
<td>0.076 ± 0.004</td>
</tr>
<tr>
<td>A. porrum L.</td>
<td>0.521 ± 0.005</td>
</tr>
<tr>
<td>A. sativum L.</td>
<td>0.043 ± 0.003</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>0.078 ± 0.006</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (bunga kuchai)</td>
<td>0.055 ± 0.006</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (daun kuchai)</td>
<td>0.270 ± 0.003</td>
</tr>
<tr>
<td><strong>20 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td>A. cepa L.</td>
<td>0.309 ± 0.004</td>
</tr>
<tr>
<td>A. fistulosum L.</td>
<td>0.100 ± 0.002</td>
</tr>
<tr>
<td>A. porrum L.</td>
<td>0.529 ± 0.005</td>
</tr>
<tr>
<td>A. sativum L.</td>
<td>0.070 ± 0.002</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>0.256 ± 0.031</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (bunga kuchai)</td>
<td>0.063 ± 0.003</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (daun kuchai)</td>
<td>0.402 ± 0.002</td>
</tr>
</tbody>
</table>

*Absorbance reading as a mean of triplicate experiments ± standard deviation (SD). SD range is not visible in graph because of the very small SD value.
Crude Chloroform Extracts

The reducing powers of chloroform *Allium* extracts in the present study are tabled in Table 4.12(b) and portrayed in Figure 4.8(b). The reducing powers of chloroform extracts at concentrations of 5 to 20 mg/ml were between 0.085A and 1.678A. Among the chloroform extracts, *A. cepa* L. and *A. fistulosum* L. exhibited moderate reducing powers when compared to ascorbic acid. Other chloroform *Allium* extracts exerted low reducing powers.

At 5 mg/ml, the reducing powers exhibited by the chloroform extracts were *A. cepa* L. > *A. fistulosum* L. > *A. porrum* L. > *A. ursinum* L. > *A. tuberosum* Rottl. (daun kuchai) > *A. tuberosum* Rottl. (bunga kuchai) > *A. sativum* L. in a decreasing manner. The reducing power of the chloroform extract of *A. cepa* L. was 1.180A±0.004 at 5 mg/ml while *A. sativum* L. exerted poor reducing power of 0.085A±0.006 at the same concentration.

At 20 mg/ml, the reducing powers of chloroform extracts of *A. cepa* L. and *A. fistulosum* L. were moderate while other *Allium* methanol extracts exerted lower reducing powers than that of ascorbic acid. Methanol extract of *A. cepa* L. exhibited the highest reducing power of 1.678A±0.01 while *A. tuberosum* Rottl. (daun kuchai) exhibited the lowest reducing power with 0.229A±0.00.
Table 4.12(b) The reducing power of various concentrations of crude chloroform extracts of selected *Allium* spp.

<table>
<thead>
<tr>
<th>Concentration of Extract (mg/ml)</th>
<th>Absorbance Reading*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.180 ± 0.004</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>0.432 ± 0.003</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.185 ± 0.008</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.085 ± 0.006</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.160 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.109 ± 0.003</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>0.112 ± 0.004</td>
</tr>
<tr>
<td><strong>10.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.538 ± 0.011</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>0.892 ± 0.013</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.327 ± 0.004</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.146 ± 0.004</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.305 ± 0.025</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.208 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>0.151 ± 0.008</td>
</tr>
<tr>
<td><strong>15.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.611 ± 0.007</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>1.442 ± 0.003</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.448 ± 0.005</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.221 ± 0.006</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.354 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.282 ± 0.007</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>0.184 ± 0.009</td>
</tr>
<tr>
<td><strong>20 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.678 ± 0.005</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>1.635 ± 0.002</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.519 ± 0.007</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.252 ± 0.002</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.535 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.348 ± 0.004</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>0.229 ± 0.003</td>
</tr>
</tbody>
</table>
Table 4.12(b) continued

*Absorbance reading as a mean of triplicate experiments ± standard deviation (SD). SD range is not visible in graph because of the very small SD value

**Crude Methanol Extracts**

As shown in Figure 4.8(c), the reducing powers of methanol *Allium* extracts were ranged between 0.140A and 2.492A for concentrations of 5 to 20 mg/ml. At all concentrations of extract (i.e. 5 to 20 mg/ml), the methanol extract of *A. fistulosum* L. exhibited the highest reducing power among the *Allium* extracts but when compared to ascorbic acid, the reducing power of this extract was moderately high. Among the *Allium* extracts, methanol extract of *A. ursinum* L. was the poorest reducing agent.

At the lowest concentration of 5 mg/ml, methanol extract of *A. fistulosum* L. was most reductive with 0.838A±0.004 followed by *A. tuberosum* Rottl. (daun kuchai) with 0.661A±0.005, *A. cepa* L. with 0.587A±0.004, *A. porrum* L. with 0.577A±0.005, *A. tuberosum* Rottl. (bunga kuchai) with 0.437A±0.002 and then *A. sativum* L. with 0.332A±0.002. The weakest reducing agent at 5 mg/ml was methanol *A. ursinum* L. extract which exhibited reducing power of 0.140A±0.002.

The methanol extract of *A. fistulosum* L. exerted moderately high reducing power when compared to ascorbic acid at the highest concentration of extract (i.e. 20 mg/ml) while methanol extracts of *A. cepa* L. and *A. porrum* L. exhibited moderate reducing powers than that of ascorbic acid at the same concentration of extract. It was observed that the methanol extract of *A. porrum* L. exerted reducing power lower than that of *A. cepa* L. at concentration 5 to 15 mg/ml but was better at 20 mg/ml. Low reducing powers were
exhibited by *A. sativum* L., *A. ursinum* L. and the two variants of *A. tuberosum* Rottl. (i.e. bunga kuchai and daun kuchai) at 20 mg/ml.

Overall results indicate that methanol extracts of the *Allium* spp. exhibited better reducing power than its petroleum ether and chloroform counterparts. The reducing powers of crude *Allium* methanol extracts in the present study are shown in Table 4.12(c).

**Table 4.12(c) The reducing power of various concentrations of crude methanol extracts of selected *Allium* spp.**

<table>
<thead>
<tr>
<th>Concentration of Extract (mg/ml)</th>
<th>Absorbance Reading*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>0.587 ± 0.004</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>0.838 ± 0.004</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.577 ± 0.005</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.332 ± 0.002</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.140 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.437 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>0.661 ± 0.005</td>
</tr>
<tr>
<td><strong>10.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.149 ± 0.010</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>1.592 ± 0.006</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.937 ± 0.001</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.482 ± 0.004</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.347 ± 0.004</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.554 ± 0.005</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>0.958 ± 0.009</td>
</tr>
<tr>
<td><strong>15.0 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.430 ± 0.007</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>2.077 ± 0.025</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>1.076 ± 0.001</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.693 ± 0.004</td>
</tr>
</tbody>
</table>
Table 4.12(c) continued

<table>
<thead>
<tr>
<th>Allium species</th>
<th>Absorbance reading (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.421 ± 0.004</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.566 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>1.044 ± 0.004</td>
</tr>
<tr>
<td><strong>20 mg/ml</strong></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.467 ± 0.006</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>2.492 ± 0.029</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>1.660 ± 0.002</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.943 ± 0.003</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.588 ± 0.002</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (bunga kuchai)</td>
<td>0.623 ± 0.005</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (daun kuchai)</td>
<td>1.090 ± 0.004</td>
</tr>
</tbody>
</table>

*Absorbance reading as a mean of triplicate experiments ± standard deviation (SD). SD range is not visible in graph because of the very small SD value.

4.4 Total Phenolic Content of Selected Allium spp.

In the present study, a total of 21 crude *Allium* extracts were evaluated for their total phenolic content. The results were summarized and presented in Table 4.13 and the total phenolic content of the crude *Allium* extracts are illustrated in Figure 4.9. Overall, the methanol *Allium* extracts contained the highest content of phenolic compounds followed by chloroform and petroleum ether extracts. However, the highest total phenolic content was exhibited by chloroform extract of *A. cepa* L. with 820 ± 0.00 mg GAEs/100 g sample while the lowest total phenolic content was found in petroleum ether *A. porrum* L. extract (i.e. 35 ± 1.53 mg GAEs/100 g sample).

Crude Petroleum Ether Extracts

The petroleum ether *Allium* extracts contained the least amount of phenolic compounds with a range of 35 mg GAEs/100 g sample to 241 mg GAEs/100 g sample. Among the petroleum ether extracts, *A. fistulosum* L. registered the highest total phenolic content of
Figure 4.8(a) Summary of the reducing power of crude petroleum ether extracts of selected *Allium* spp.
Figure 4.8(b) Summary of the reducing power of crude chloroform extracts of selected *Allium* spp.
Figure 4.8(c) Summary of the reducing power of crude methanol extracts of selected *Allium* spp.
241±1.00 mg GAEs/100 g sample while *A. porrum* L. contained lowest total phenolic content of 35±1.53 mg GAEs/100 g sample.

**Crude Chloroform Extracts**

Overall, the total phenolic content found in the chloroform *Allium* extracts was average. The total phenolic content of chloroform ranges from 58 mg GAEs/100 g sample to 820 mg GAEs/100 g sample. Among the chloroform extracts, *A. cepa* L. stood out with the highest total phenolic content of 820±0.00 mg GAEs/100 g sample. The chloroform extract of *A. tuberosum* Rottl. (daun kuchai) contained high amount of total phenolic content of 356±0.00 mg GAEs/100 g sample and the least total phenolic content was found in the other variant, *A. tuberosum* Rottl. (bunga kuchai) with 58±1.00 mg GAEs/100 g sample.

**Crude Methanol Extracts**

Based on the results, the methanol extracts in the present study was found to exhibit the higher total phenolic content when compared with its petroleum ether and chloroform counterparts. The total phenolic content of the methanol *Allium* extracts ranged from 111 mg GAEs/100 g sample to 365 mg GAEs/100 g sample. The highest total phenolic content for methanol extracts was exerted by *A. fistulosum* L. with 365±0.00 mg GAEs/100 g sample while the lowest total phenolic content was expressed by *A. ursinum* L. with 111 ± 2.52 mg GAEs/100 g sample.
Table 4.13 Total phenolic content of selected *Allium* spp. as determined using the Folin-Ciocalteau assay

<table>
<thead>
<tr>
<th>Concentration of Crude Extract (20 mg/ml)</th>
<th>Total Phenolic Content in Crude Extract* (mg GAEs/100 g sample) y = 0.001x</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum Ether</td>
</tr>
<tr>
<td><em>A. cepa L.</em></td>
<td>104 ± 2.52</td>
</tr>
<tr>
<td><em>A. fistulosum L.</em></td>
<td>241 ± 1.00</td>
</tr>
<tr>
<td><em>A. porrum L.</em></td>
<td>35 ± 1.53</td>
</tr>
<tr>
<td><em>A. sativum L.</em></td>
<td>54 ± 1.15</td>
</tr>
<tr>
<td><em>A. ursinum L.</em></td>
<td>149 ± 2.00</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>52 ± 1.00</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>38 ± 2.00</td>
</tr>
</tbody>
</table>

* Total phenolic content in crude extracts as a mean of triplicate experiments ± standard deviation (SD). SD range not visible in graph because of the small SD value.

4.5 Total Flavonoid Content of Selected *Allium* spp.

A total of 21 crude *Allium* extracts were evaluated for their total flavonoid content. The results were summarized and presented in Table 4.14 and the total flavonoid content of the crude extracts are illustrated in Figure 4.10. Overall results indicated that the chloroform *Allium* extracts exhibited total flavonoid content higher than its petroleum ether and methanol extracts. The highest total flavonoid content was exhibited by methanol *A. porrum* L. extract with 56.48±0.00 mg QEs/100 g sample while the lowest total flavonoid content was exerted by the methanol extract of *A. tuberosum* Rottl. (daun kuchai) with 3.60±0.00 mg QEs/100 g sample.

Crude Petroleum Ether Extracts

Based on the results, the total flavonoid content for petroleum ether *Allium* extracts ranged between 7.25 mg QEs/100 g sample and 32.93 mg QEs/100 g sample. Of the
Figure 4.9 Total phenolic content of selected *Allium* spp. expressed as mg GAEs/100 g sample
petroleum ether extracts, the highest total flavonoid content was exerted by *A. tuberosum* Rottl. (bunga kuchai) with 32.92±0.01 mg QEs/100 g sample but the *A. tuberosum* Rottl. *daun* variant demonstrated otherwise. The *daun* variant exhibited the lowest total flavonoid content of 7.25±0.00 mg QEs/100 g sample.

**Crude Chloroform Extracts**

In general, the range of total flavonoid content of chloroform *Allium* extracts was between 8.02 mg QEs/100 g sample and 23.60 mg QEs/100 g sample. Among the chloroform extracts, *A. cepa* L. contained the highest total flavonoid content of 23.60±0.01 mg QEs/100 g sample while the lowest total flavonoid content was found in chloroform extracts of *A. porrum* L. with 8.02±0.00 mg QEs/100 g sample.

**Crude Methanol Extracts**

Overall, the total flavonoid content of methanol extracts ranged from 3.60 QEs/100 g sample to 56.48 mg QEs/100 g sample. Of the methanol extracts, the highest total flavonoid content was exhibited by *A. porrum* L. with 56.48±0.00 mg QEs/100 g sample. *A. ursinum* L. exhibited the lowest total flavonoid content of 3.60±0.00 mg QEs/100 g sample.
Table 4.14 Total flavonoid content of selected *Allium* spp. as determined using the AlCl$_3$ colorimetric assay

<table>
<thead>
<tr>
<th>Concentration of Crude Extract (20 mg/ml)</th>
<th>Total Flavonoid Content in Crude Extract* (mg QEs/100 g sample) y = 0.0104x - 0.0004</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum Ether</td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>13.88 ± 0.01</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>14.85 ± 0.01</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>27.25 ± 0.00</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>8.12 ± 0.00</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>11.87 ± 0.00</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>32.92 ± 0.01</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>7.25 ± 0.00</td>
</tr>
</tbody>
</table>

* Total flavonoid content in crude extracts as a mean of triplicate experiments ± standard deviation (SD). SD range not visible in graph because of the small SD value.

4.6 Antioxidant Activities of Selected *Allium* spp. and Their Total Phenolic and Flavonoid Contents

In the present study, the results of the antioxidant activities of the *Allium* plants were compared to evaluate the possibility of a relationship between their antioxidant activity and total phenolic and flavonoid contents. However, no exact correlation was observed in between the antioxidant activities of the *Allium* crude extracts and their total phenolic and flavonoid contents.

To simplify the comparison process, Vimala and Ilham (1999) had categorized antioxidant activity as high with $\geq 70\%$ activity, moderate with $\geq 50\%$ activity and low with $< 50\%$ activity. For the purpose of evaluating the relationship of antioxidant activity and total phenolic and flavonoid contents in the present study, the following status were used (Table 4.15).
Figure 4.10 Total flavonoid content of selected *Allium* spp. expressed as mg QEs/100 g sample
Table 4.15 Antioxidant activity and total phenolic and flavonoid contents status

<table>
<thead>
<tr>
<th>Antioxidant Activity Status</th>
<th>Reducing Power (A)</th>
<th>Total Phenolic Content (mg GAEs/100 g sample)</th>
<th>Total Flavonoid Content (mg QEs/100 g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (≥ 70% activity)</td>
<td>≥ 2.100A</td>
<td>≥ 255</td>
<td>≥ 23</td>
</tr>
<tr>
<td>Moderate (≥ 50% activity)</td>
<td>≥ 1.500A</td>
<td>≥ 183</td>
<td>≥ 16</td>
</tr>
<tr>
<td>Low (&lt; 50% activity)</td>
<td>&lt; 1.500A</td>
<td>&lt; 183</td>
<td>&lt; 16</td>
</tr>
</tbody>
</table>

In the present study, a positive correlation means high antioxidant activity registered alongside high content of total phenolic and/or flavonoid content. A negative correlation is reflected by a low antioxidant activity registered alongside low content of total phenolic and/or flavonoid content. An inverse relationship means high or low antioxidant activity alongside low or high total phenolic and/or flavonoid content.

4.6.1 β-Carotene Bleaching Inhibition Activity and Total Phenolic and Flavonoid Contents

The relationship between the β-carotene bleaching inhibition activities of crude *Allium* spp. extracts and their total phenolic and flavonoid contents is shown in Table 4.16.

**β-Carotene Bleaching Inhibition Activity and Total Phenolic Content**

Generally, only one *Allium* extract had demonstrated positive correlation between the β-carotene bleaching inhibition activity and their total phenolic content. The methanol extract of *A. sativum* L. showed positive relationship by exhibiting high antioxidant activity of 77.37% ± 2.51 and high total phenolic content of 251 ± 1.00 mg GAEs/100 g sample.
42.86% of the *Allium* extracts exhibited an inverse β-carotene bleaching inhibition activity and total phenolic content relationship. The petroleum ether extracts of *A. cepa* L., *A. porrum* L. and *A. tuberosum* Rottl. (daun kuchai) had high antioxidant activities when their total phenolic contents was low. For example, the petroleum ether extract of *A. cepa* L. exhibited high inhibition activity of 91.10%±3.71 but contained low total phenolic content of 104±2.52 mg GAEs/100 g sample.

This inverse relationship was also observed in chloroform *A. cepa* L. and methanol extracts of *A. fistulosum* L. and *A. tuberosum* Rottl. (daun kuchai). These extracts demonstrated low β-carotene bleaching inhibition activities with high total phenolic contents. *Allium cepa* L. exhibited low antioxidant activity of 34.18%±4.01 but exerted highest total phenolic content with 820±0.00 mg GAEs/100 g sample.

Overall, the highest β-carotene bleaching inhibition activity was shown by *A. cepa* L. petroleum ether extract with 91.10%±3.71 but this extract exhibited only low total phenolic content of 104±2.52 mg GAEs/100 g sample. The lowest β-carotene bleaching inhibition activity was observed in petroleum ether *A. ursinum* L. extract with 25.20%±2.51 with a low total phenolic content of 149±2.00 mg GAEs/100 g sample.

**β-Carotene Bleaching Inhibition Activity and Total Flavonoid Content**

Results show that there was no positive correlation between the antioxidant activity of the *Allium* extracts and their total flavonoid content. 19.05% of the *Allium* extracts exhibited a negative correlation between their β-carotene bleaching inhibition activities and total flavonoid content. The petroleum ether *A. ursinum* L. extract and methanol
extracts of *A. ursinum* L. and both variants of *A. tuberosum* Rottl. gave low β-carotene bleaching inhibition activities with low total flavonoid contents.

Other 23.81% of *Allium* extracts demonstrated an inverse relationship between their β-carotene bleaching inhibition activities and total flavonoid contents. The petroleum ether extract of *A. cepa* L. and *A. tuberosum* Rottl. (daun kuchai) and methanol extract of *A. sativum* L. exhibited high antioxidant activities with low total flavonoid contents. For instance, the petroleum ether *A. cepa* L. extract exhibited the highest antioxidant activity of 91.10%±3.71 but contained low total flavonoid content of 13.88 ± 0.00 mg QEs/100 g sample.

On the other hand, chloroform extract of *A. cepa* L. and methanol extract of *A. porrum* L. exhibited low antioxidant activities with high total flavonoid contents. The methanol extract of *A. porrum* L. exhibited low antioxidant activity of 32.78%±4.01 with highest total flavonoid content of 56.48 ± 0.00 mg QEs/100 g sample.

Generally, results show that the *A. cepa* L. petroleum ether extract exhibited the highest β-carotene bleaching inhibition activity of 91.10%±3.71 but with low total flavonoid content of 13.88 ± 0.00 mg QEs/100 g sample. The lowest β-carotene bleaching inhibition activity was demonstrated by petroleum ether *A. ursinum* L. extract with 25.20%±2.51 at the concentration of 20 mg/ml extract and its total flavonoid content was low with 11.87 ± 0.00 mg QEs/100 g sample.
<table>
<thead>
<tr>
<th>Plant Antioxidant Activity* (%)</th>
<th>Total Phenolic Content* (mg GAEs/ 100 g sample)</th>
<th>Total Flavonoid Content* (mg QEs/ 100 g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Extract</td>
<td>20 mg/ml</td>
<td>20 mg/ml</td>
</tr>
<tr>
<td>Petroleum Ether</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. cepa L.</td>
<td>91.10±3.71</td>
<td>104 ± 2.52</td>
</tr>
<tr>
<td>A. fistulosum L.</td>
<td>56.76±5.19</td>
<td>241 ± 1.00</td>
</tr>
<tr>
<td>A. porrum L.</td>
<td>86.04±9.97</td>
<td>35 ± 1.53</td>
</tr>
<tr>
<td>A. sativum L.</td>
<td>66.21±4.31</td>
<td>54 ± 1.15</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>25.20±2.51</td>
<td>149 ± 2.00</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (Bunga kuchai)</td>
<td>66.01±2.93</td>
<td>52 ± 1.00</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (Daun kuchai)</td>
<td>74.91±1.95</td>
<td>38 ± 2.00</td>
</tr>
<tr>
<td>Chloroform</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. cepa L.</td>
<td>34.18±4.01</td>
<td>820 ± 0.00</td>
</tr>
<tr>
<td>A. fistulosum L.</td>
<td>54.48±3.68</td>
<td>242 ± 2.52</td>
</tr>
<tr>
<td>A. porrum L.</td>
<td>89.00±5.08</td>
<td>173 ± 2.65</td>
</tr>
<tr>
<td>A. sativum L.</td>
<td>71.47±3.63</td>
<td>152 ± 1.53</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>90.77±2.01</td>
<td>112 ± 3.06</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (Bunga kuchai)</td>
<td>67.58±2.71</td>
<td>58 ± 1.00</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (Daun kuchai)</td>
<td>58.46±1.13</td>
<td>356 ± 0.00</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. cepa L.</td>
<td>61.78±4.09</td>
<td>168 ± 1.53</td>
</tr>
<tr>
<td>A. fistulosum L.</td>
<td>26.48±0.89</td>
<td>365 ± 0.00</td>
</tr>
<tr>
<td>A. porrum L.</td>
<td>32.78±4.01</td>
<td>256 ± 1.00</td>
</tr>
<tr>
<td>A. sativum L.</td>
<td>77.37±2.51</td>
<td>251 ± 1.00</td>
</tr>
<tr>
<td>A. ursinum L.</td>
<td>43.66±1.01</td>
<td>111 ± 2.52</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (Bunga kuchai)</td>
<td>44.36±3.92</td>
<td>245 ± 1.53</td>
</tr>
<tr>
<td>A. tuberosum Rottl. (Daun kuchai)</td>
<td>43.99±1.31</td>
<td>261 ± 2.31</td>
</tr>
</tbody>
</table>
4.6.2 Lipid Peroxidation Inhibition Activity and Total Phenolic and Flavonoid Contents

The relationship between the antioxidant activities of crude *Allium* spp. extracts as determined by the FTC assay and their total phenolic and flavonoid contents is shown in Table 4.17. The antioxidant activities of the *Allium* samples were compared based on their antioxidant activities recorded on the eighth day of the experiment. Lipid peroxidation was taken as 100% on that day (Emami *et al.*, 2007).

**Lipid Peroxidation Inhibition Activity and Total Phenolic Content**

Results indicated that 4.76% and 23.81% of the crude *Allium* extracts respectively showed positive and negative correlation between their lipid peroxidation (LPO) inhibition activities and total phenolic contents. No correlation was seen for other *Allium* extracts. The chloroform extract of *A. tuberosum* Rottl. (daun kuchai) exhibited positive correlation with high antioxidant activity of 85.65%±0.08 with high total phenolic content of 356±0.00 mg GAEs/100 g sample while negative correlation was demonstrated by petroleum ether extracts of *A. cepa* L., *A. sativum* L., *A. ursinum* L., chloroform extract of *A. sativum* L. and methanol extracts of *A. cepa* L. and *A. ursinum* L.. For instance, the petroleum ether extract of *A. sativum* L. exhibited 5.99%±0.28 LPO inhibition activity and low 54±1.15 mg GAEs/100 g sample total phenolic content.
33.33% of the Allium extracts exhibited an inverse relationship between their LPO inhibition activities and total phenolic contents. For instance, the petroleum ether extract of A. tuberosum Rottl. (daun kuchai) and chloroform extracts of A. porrum L., A. ursinum L. and A. tuberosum Rottl. (bunga kuchai) extract demonstrated high antioxidant activities but contained low total phenolic contents. Likewise, methanol extracts of A. fistulosum L., A. porrum L. and A. tuberosum Rottl. (daun kuchai) demonstrated low antioxidant activities but with high content of phenolics.

Methanol extracts of A. fistulosum L. A. porrum L. and A. tuberosum Rottl. (daun kuchai) respectively exhibited low antioxidant activities of 4.02%±0.37, 7.51%±1.60 and 27.57%±0.05 but with high total phenolic contents of 365±0.00 mg GAEs/100 g sample, 256±1.00 mg GAEs/100 g sample and 261±2.31 mg GAEs/100 g sample.

In general, the highest LPO inhibition activity of 86.17%±0.16 was exhibited by A. fistulosum L. chloroform extract but this extract contained moderate phenolics amounts of 242±2.52 mg GAEs/100 g sample. The weakest 4.02%±0.37 LPO inhibition was exerted by methanol A. fistulosum L. extract containing high total phenolic content of 365±0.00 mg GAEs/100 g sample.

Lipid Peroxidation Inhibition Activity and Total Flavonoid Content

Overall results indicate no positive correlation between the antioxidant activities and total flavonoid contents of the Allium extracts. However, seven Allium extracts showed a negative correlation between their antioxidant activities and total flavonoid contents. The petroleum ether extracts of A. cepa L., A. sativum L. and A. ursinum L. and
methanol extracts of *A. sativum* L., *A. ursinum* L. and both variants of *A. tuberosum* Rottl. showed negative correlation between their antioxidant activities and total flavonoid contents. These extracts exhibited low LPO inhibition activities when their total flavonoid contents were also low.

19.05% of the *Allium* extracts exhibited an inverse relationship between antioxidant activities and total flavonoid contents. As seen, petroleum ether and chloroform extracts of *A. fistulosum* L. and *A. tuberosum* Rottl. (daun kuchai) exhibited high antioxidant activities when their contents of flavonoids were low. Although the *A. porrum* L. methanol extract had exhibited low LPO inhibition of 7.51%±1.60, this extract contained high amounts of flavonoids (i.e. 56.48±0.00 mg QEs/100 g sample).

Generally, the chloroform *A. fistulosum* L. extract exhibited the highest antioxidant activity of 86.17%±0.16 but contained low total flavonoid content of 8.02±0.00 mg QEs/100 g sample. *A. fistulosum* L. methanol extract exhibited lowest LPO inhibition activity when its total flavonoid content was a moderate 18.02±0.00 mg QEs/100 g sample.
Table 4.17 The relationship between antioxidant activities of crude *Allium* spp. extracts determined by the FTC assay and their total phenolic and flavonoid contents

<table>
<thead>
<tr>
<th>Concentration of Extract</th>
<th>Plant</th>
<th>Antioxidant Activity*(%)(Day 8)</th>
<th>Total Phenolic Content* (mg GAEs/100 g sample)</th>
<th>Total Flavonoid Content* (mg QEs/100 g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.2 mg/ml</td>
<td>20 mg/ml</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>Petroleum Ether</td>
<td><em>A. cepa</em> L.</td>
<td>26.10±0.68</td>
<td>104 ± 2.52</td>
<td>13.88 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. fistulosum</em> L.</td>
<td>83.33±0.11</td>
<td>241 ± 1.00</td>
<td>14.85 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. porrum</em> L.</td>
<td>60.00±0.13</td>
<td>35 ± 1.53</td>
<td>27.25 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. sativum</em> L.</td>
<td>5.99±0.28</td>
<td>54 ± 1.15</td>
<td>8.12 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. ursinum</em> L.</td>
<td>28.50±0.24</td>
<td>149 ± 2.00</td>
<td>11.87 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>51.33±0.67</td>
<td>52 ± 1.00</td>
<td>32.92 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>83.70±0.34</td>
<td>38 ± 2.00</td>
<td>7.25 ± 0.00</td>
</tr>
<tr>
<td>Chloroform</td>
<td><em>A. cepa</em> L.</td>
<td>68.38±0.30</td>
<td>820 ± 0.00</td>
<td>23.60 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. fistulosum</em> L.</td>
<td>86.17±0.16</td>
<td>242 ± 2.52</td>
<td>8.02 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. porrum</em> L.</td>
<td>85.17±0.21</td>
<td>173 ± 2.65</td>
<td>19.08 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. sativum</em> L.</td>
<td>15.30±0.40</td>
<td>152 ± 1.53</td>
<td>17.83 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. ursinum</em> L.</td>
<td>71.25±0.23</td>
<td>112 ± 3.06</td>
<td>17.44 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>84.95±0.32</td>
<td>58 ± 1.00</td>
<td>22.54 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>85.65±0.08</td>
<td>356 ± 0.00</td>
<td>13.98 ± 0.00</td>
</tr>
<tr>
<td>Methanol</td>
<td><em>A. cepa</em> L.</td>
<td>13.32±0.78</td>
<td>168 ± 1.53</td>
<td>19.85 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. fistulosum</em> L.</td>
<td>4.02±0.37</td>
<td>365 ± 0.00</td>
<td>18.02 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. porrum</em> L.</td>
<td>7.51±1.60</td>
<td>256 ± 1.00</td>
<td>56.48 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. sativum</em> L.</td>
<td>26.63±0.71</td>
<td>251 ± 1.00</td>
<td>7.06 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. ursinum</em> L.</td>
<td>36.56±0.50</td>
<td>111 ± 2.52</td>
<td>10.13 ± 0.01</td>
</tr>
<tr>
<td></td>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>12.82±6.82</td>
<td>245 ± 1.53</td>
<td>11.10 ± 0.00</td>
</tr>
<tr>
<td></td>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>27.57±0.05</td>
<td>261 ± 2.31</td>
<td>3.60 ± 0.00</td>
</tr>
</tbody>
</table>
Table 4.17 continued

* Antioxidant activity and total phenolic and flavonoid contents in crude extracts as a mean of triplicate experiments ± standard deviation (SD). Please refer pg. 159 for description of colour code

4.6.3 DPPH Radical Scavenging Activity and Total Phenolic and Flavonoid Contents

The relationship between the DPPH radical scavenging activities of crude Allium spp. extracts and their total phenolic and flavonoid contents is shown in Table 4.18.

DPPH Radical Scavenging Activity and Total Phenolic Content

Based on the results, 4.76% of the Allium extracts showed positive correlation between DPPH radical scavenging activities and total phenolic contents. Chloroform A. cepa L. extract exhibited highest scavenging activity of 86.27%±0.05 and contained highest amounts of phenolics (i.e. 820±0.00 mg GAEs/100 g sample). 47.62% of the extracts exhibited negative correlation where these extracts exhibited low antioxidant activities and had low content of phenolics. Antioxidant activities exhibited by all petroleum ether extracts except A. fistulosum L. and A. tuberosum Rottl. (daun kuchai), chloroform extracts of A. porrum L., A. sativum L. and A. tuberosum Rottl. (bunga kuchai) and methanol extracts of A. cepa L. and A. ursinum L. showed negative correlation with their total phenolic contents.

Other 23.81% of Allium extracts exhibited an inverse relationship between their DPPH radical scavenging activities and total phenolic contents. Although the chloroform A. tuberosum Rottl. (daun kuchai) extract and methanol A. fistulosum L., A. porrum L., A.
*sativum* L. and *A. tuberosum* Rottl. (daun kuchai) extracts exhibited low antioxidant activities, they have high content of phenolics.

For instance, chloroform *A. tuberosum* Rottl. (daun kuchai) extract had low DPPH radical scavenging activity of 13.90±0.08 but its total phenolic content was among the highest with 356±0.00 mg GAEs/100 g sample. On the other hand, chloroform extract of *A. ursinum* L. exhibited high antioxidant activity but with low total phenolic content of 112±3.06 mg GAEs/100 g sample.

In general, results showed that the highest antioxidant activity 86.27%±0.05 was exerted by chloroform extract of *A. cepa* L. with highest total phenolic content of 820±0.00 mg GAEs/100 g sample. The weakest DPPH radical scavenger was petroleum ether *A. porrum* L. extract with percentage of inhibition of 1.56%±0.13 and lowest total phenolic content of 35±1.53 mg GAEs/100 g sample.

**DPPH Radical Scavenging Activity and Total Flavonoid Content**

As shown in Table 4.18, chloroform extract of *A. cepa* L. exhibited positive correlation between its DPPH radical scavenging activity and total flavonoid content. This extract gave DPPH inhibition activity of 86.27%±0.05 with high total flavonoid content of 23.60±0.01 mg QEs/100 g sample. *Allium* extracts showed negative correlation between the DPPH radical scavenging activities and their total flavonoid contents.

exerted low scavenging activities and low total flavonoid contents. *Allium ursinum* chloroform extract gave high scavenging activity with moderate total flavonoid contents. In addition to that, methanol extracts of *A. sativum* L., *A. ursinum* L. and both variants of *A. tuberosum* Rottl. also demonstrated negative correlation between their antioxidant activities and total flavonoid contents.

An inverse relationship was observed between the DPPH radical scavenging activities and total flavonoid contents in 14.29% of the *Allium* extracts. For instance, petroleum ether extracts *A. tuberosum* Rottl. (bunga kuchai) exhibited low DPPH radical scavenging activity of 26.97%±0.08 with high total flavonoid content of 32.92±0.01 mg QEs/100 g sample. Likewise, methanol extract of *A. porrum* L. exhibited weak antioxidant activity of 2.22%±0.00 but had the highest total flavonoid content of 56.48±0.00 mg QEs/100 g sample.

Overall it was observed that the highest DPPH radical scavenging activity was exhibited by chloroform extract of *A. cepa* L. with 86.27%±0.05 with high amounts of flavonoids (i.e. 23.60±0.01 mg QEs/100 g sample). The weakest DPPH inhibition activity of 1.56%±0.13 was exerted by petroleum ether *A. porrum* L. extract which exhibited high total flavonoid content of 27.25±0.00 mg QEs/100 g sample.
Table 4.18 The relationship between DPPH radical scavenging activities of crude *Allium* spp. extracts and their total phenolic and flavonoid contents

<table>
<thead>
<tr>
<th>Concentration of Extract</th>
<th>Plant Antioxidant Activity*(%)</th>
<th>Total Phenolic Content* (mg GAEs/100g sample)</th>
<th>Total Flavonoid Content* (mg QEs/100g sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 mg/ml</td>
<td>20 mg/ml</td>
</tr>
<tr>
<td>Petroleum Ether</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>4.11±0.08</td>
<td>104 ± 2.52</td>
<td>13.88 ± 0.00</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>44.86±0.05</td>
<td>241 ± 1.00</td>
<td>14.85 ± 0.00</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>1.56±0.13</td>
<td>35 ± 1.53</td>
<td>27.25 ± 0.00</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>11.92±0.00</td>
<td>54 ± 1.15</td>
<td>8.12 ± 0.00</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>5.84±0.08</td>
<td>149 ± 2.00</td>
<td>11.87 ± 0.00</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>26.97±0.08</td>
<td>52 ± 1.00</td>
<td>32.92 ± 0.01</td>
</tr>
<tr>
<td>(Bunga kuchai)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>51.23±0.00</td>
<td>38 ± 2.00</td>
<td>7.25 ± 0.00</td>
</tr>
<tr>
<td>(Daun kuchai)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>86.27±0.05</td>
<td>820 ± 0.00</td>
<td>23.60 ± 0.01</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>26.44±0.00</td>
<td>242 ± 2.52</td>
<td>8.02 ± 0.00</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>21.38±0.05</td>
<td>173 ± 2.65</td>
<td>19.08 ± 0.01</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>10.69±0.05</td>
<td>152 ± 1.53</td>
<td>17.83 ± 0.01</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>72.45±0.00</td>
<td>112 ± 3.06</td>
<td>17.44 ± 0.00</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>19.57±0.00</td>
<td>58 ± 1.00</td>
<td>22.54 ± 0.01</td>
</tr>
<tr>
<td>(Bunga kuchai)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>13.90±0.08</td>
<td>356 ± 0.00</td>
<td>13.98 ± 0.00</td>
</tr>
<tr>
<td>(Daun kuchai)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>8.72±0.05</td>
<td>168 ± 1.53</td>
<td>19.85 ± 0.01</td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>15.70±0.00</td>
<td>365 ± 0.00</td>
<td>18.02 ± 0.00</td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>2.22±0.00</td>
<td>256 ± 1.00</td>
<td>56.48 ± 0.00</td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>14.72±0.05</td>
<td>251 ± 1.00</td>
<td>7.06 ± 0.00</td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>7.07±0.08</td>
<td>111 ± 2.52</td>
<td>10.13 ± 0.01</td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>13.82±0.05</td>
<td>245 ± 1.53</td>
<td>11.10 ± 0.00</td>
</tr>
<tr>
<td>(Bunga kuchai)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>12.42±0.00</td>
<td>261 ± 2.31</td>
<td>3.60 ± 0.00</td>
</tr>
<tr>
<td>(Daun kuchai)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.18 continued

* Antioxidant activity and/or total phenolic and flavonoid contents in crude extracts as a mean of triplicate experiments ± standard deviation (SD). Please refer pg. 159 for description of colour code

4.6.4 Reducing Powers and Total Phenolic and Total Flavonoid Contents

The relationship between the reducing powers of crude *Allium* spp. extracts and their total phenolic and flavonoid contents is shown in Table 4.19.

Reducing Powers and Total Phenolic Content

As shown in Table 4.19, one *Allium* extract showed positive correlation between its reducing power and total phenolic content. The methanol extract of *A. fistulosum* L. exhibited high reducing power of 2.492±0.03 and high total phenolic contents of 365±0.00 mg GAEs/100 g sample.

57.14% of the *Allium* extracts showed negative correlation in their reducing powers and total phenolic contents. The *Allium* extracts that gave negative correlation were all petroleum ether extracts except *A. fistulosum* L., chloroform extracts of *A. porrum* L., *A. sativum* L., *A. ursinum* L. and *A. tuberosum* Rottl. (bunga kuchai) and methanol extracts of *A. cepa* L. and *A. ursinum* L.. These extract all exhibited low reducing powers with low total phenolic contents. For instance, petroleum ether extract of *A. tuberosum* Rottl. (bunga kuchai) exhibited lowest reducing power of 0.063±0.00 and also low total phenolic content of 52±1.00 mg GAEs/100 g sample.
Among the extracts, only the chloroform and methanol extracts of *A. tuberosum* Rottl. (daun kuchai) showed inverse relationship in its reducing power and total phenolic content. Although both chloroform and methanol extracts of *daun kuchai* exhibited weak reducing powers, nonetheless their total phenolic contents were among the highest with 356±0.00 mg GAEs/100 g sample and 261±2.31 mg GAEs/100 g sample respectively.

Generally, the highest reducing power was exhibited by methanol *A. fistulosum* L. extract with 2.492±0.03 and contained a high total phenolic content of 365±0.00 mg GAEs/100 g sample. The weakest reducing power was observed in petroleum ether extract of *A. tuberosum* Rottl. (bunga kuchai) with 0.063±0.00 and a low 52±1.00 mg GAEs/100 g sample total phenolic content.

Reducing Powers and Total Flavonoid Content

Based on the results, no positive correlation was found between the reducing powers and the total flavonoid contents of the *Allium* extracts. 47.62% of the *Allium* extracts in the present study showed negative correlation in their reducing powers and total flavonoid contents.

The petroleum ether extracts of *A. cepa* L., *A. fistulosum* L., *A. sativum* L., *A. ursinum* L. and *A. tuberosum* Rottl. (daun kuchai) and chloroform extracts of *A. tuberosum* Rottl. (daun kuchai) exhibited low reducing powers and also low total flavonoid contents. The same negative correlation was also seen in methanol extracts of *A. sativum* L., *A. ursinum* L. and both variants of *A. tuberosum* Rottl.. An inverse relationship between
the reducing powers and total flavonoid contents was observed for 9.52% of the *Allium* extracts. The petroleum ether extracts of *A. porrum* L. and *A. tuberosum* Rottl. (bunga kuchai) exhibited low reducing powers of 0.529±0.01 and 0.063±0.00 respectively with high total flavonoid contents of 27.25±0.00 mg QEs/100 g sample and 32.92±0.01 mg QEs/100 g sample.

Overall, the highest reducing power was exhibited by methanol *A. fistulosum* L. extract with 2.492±0.03 but its total flavonoid content was a moderate 18.02±0.00 mg QEs/100 g sample. Methanol extract of *A. porrum* L. exhibited the moderate reducing power of 1.660±0.00 and highest total flavonoid content of 56.48±0.00 mg QEs/100 g sample.

Table 4.19 The relationship between reducing powers of crude *Allium* spp. extracts and their total phenolic and flavonoid contents

<table>
<thead>
<tr>
<th>Concentration of Extract</th>
<th>Plant</th>
<th>Absorbance Reading*</th>
<th>Total Phenolic Content*</th>
<th>Total Flavonoid Content*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 mg/ml</td>
<td>20 mg/ml</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td><strong>Petroleum Ether</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>0.309 ± 0.00</td>
<td>104 ± 2.52</td>
<td>13.88 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>0.100 ± 0.00</td>
<td>241 ± 1.00</td>
<td>14.85 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.529 ± 0.01</td>
<td>35 ± 1.53</td>
<td>27.25 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.070 ± 0.00</td>
<td>54 ± 1.15</td>
<td>8.12 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.256 ± 0.03</td>
<td>149 ± 2.00</td>
<td>11.87 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Bunga kuchai)</td>
<td>0.063 ± 0.00</td>
<td>52 ± 1.00</td>
<td>32.92 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl. (Daun kuchai)</td>
<td>0.402 ± 0.00</td>
<td>38 ± 2.00</td>
<td>7.25 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Table 4.19 continued</td>
<td></td>
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</tr>
<tr>
<td>----------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chloroform</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.678 ± 0.01</td>
<td>820 ± 0.00</td>
<td>23.60 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>1.635 ± 0.00</td>
<td>242 ± 2.52</td>
<td>8.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>0.519 ± 0.01</td>
<td>173 ± 2.65</td>
<td>19.08 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.252 ± 0.00</td>
<td>152 ± 1.53</td>
<td>17.83 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.535 ± 0.00</td>
<td>112 ± 3.06</td>
<td>17.44 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>0.348 ± 0.00</td>
<td>58 ± 1.00</td>
<td>22.54 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>(Bunga kuchai)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>0.229 ± 0.00</td>
<td>356 ± 0.00</td>
<td>13.98 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>(Daun kuchai)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Methanol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. cepa</em> L.</td>
<td>1.467 ± 0.01</td>
<td>168 ± 1.53</td>
<td>19.85 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>A. fistulosum</em> L.</td>
<td>2.492 ± 0.03</td>
<td>365 ± 0.00</td>
<td>18.02 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. porrum</em> L.</td>
<td>1.660 ± 0.00</td>
<td>256 ± 1.00</td>
<td>56.48 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. sativum</em> L.</td>
<td>0.943 ± 0.00</td>
<td>251 ± 1.00</td>
<td>7.06 ± 0.00</td>
<td></td>
</tr>
<tr>
<td><em>A. ursinum</em> L.</td>
<td>0.588 ± 0.00</td>
<td>111 ± 2.52</td>
<td>10.13 ± 0.01</td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>0.623 ± 0.01</td>
<td>245 ± 1.53</td>
<td>11.10 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>(Bunga kuchai)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. tuberosum</em> Rottl.</td>
<td>1.090 ± 0.00</td>
<td>261 ± 2.31</td>
<td>3.60 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>(Daun kuchai)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Absorbance reading and total phenolic and flavonoid contents in crude extracts as a mean of triplicate experiments ± standard deviation (SD). Please refer pg. 159 for description of colour code

4.7 Evaluation of the Anti-HPV Type 16 E6 Oncoprotein Activities

In the present study, 21 crude extracts (i.e. petroleum ether, chloroform and methanol extracts) obtained from seven *Allium* plants selected from the local market were screened for possible anti-HPV type 16 E6 activity. The human epidermal carcinoma of cervix HPV type 16-containing cell line, CaSki was pretreated with various concentrations of extracts (i.e. 1 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml) for 72 hours at 37°C before subjected to ICC staining and evaluations. Microscopic images were recorded using the light microscope at 40X.
The three-step indirect Labelled Streptavidin-Biotin® 2 system was applied to detect and evaluate the anti-HPV type 16 E6 activity of these crude extracts. The anti-HPV type 16 E6 monoclonal antibody was used to detect the E6 oncoprotein expressed by the untreated and pre-treated CaSki cells. In general, the appearance of a reddish-brown stain in the cytoplasm region of the CaSki cells indicate the presence of E6 oncoprotein.

The higher the E6 expression levels in the CaSki cells, the more intense the reddish-brown stain. The enlarged microscopic images in Plate 4.1 show different intensities of reddish-brown stain. They are classified as no stain (-), mild (+), moderate (++) , strong (+++) and very strong (++++). The suppressing effect of the extracts is considered as very weak for (++++), weak for (+++), moderate for (++), strong for (+) and very strong for (-).

In the present study, there were two types of negative controls. They were CaSki cells untreated with plant’s crude extract and not incubated with anti-HPV type 16 E6 monoclonal antibody and CaSki cells untreated with plant’s crude extract but incubated with anti-HPV type 16 E6 monoclonal antibody (Plate 4.2(a-b)). As observed in Plate 4.2(a), no reddish-brown stain was seen on the negative control that was not incubated with the monoclonal antibody. On the other hand, intense reddish-brown stain was observed in cytoplasm region of the CaSki cells incubated with the monoclonal antibody as shown in Plate 4.2(b).
Plate 4.1 The classifications of reddish-brown stain intensity that indicate the presence of E6 oncoprotein in CaSki cells after ICC

Plate 4.2(a) Untreated CaSki cells without anti-HPV type 16 E6 monoclonal antibody incubation (40X); and (b) Untreated CaSki cells with anti-HPV type 16 E6 monoclonal antibody incubation (40X)
To determine and evaluate the anti-HPV type 16 E6 activities of the crude extracts, the reddish-brown staining intensities were compared among the crude extracts at each concentration of extract and also with the negative control. Concurrently, the morphological characteristics of the CaSki cells were also analyzed. Only the appearances of reddish-brown stains in viable cells are considered (Plate 4.3).

Plate 4.3 Appearance of viable cells and dead and/or dying cells after ICC

4.7.1 Anti-HPV Type 16 E6 Activities of Selected *Allium* spp.

*Allium cepa* L.

As shown in Figure 4.11, the reddish-brown stains in viable CaSki cells treated with crude extracts derived from *A. cepa* L. decreased with the increasing concentrations of plant extracts. This suggests that when tested against the CaSki cell line, all three crude extracts of this *Allium* vegetable exhibited positive anti-HPV type 16 E6 oncoprotein activities in a dose-dependent manner where extracts at higher concentrations produced greater suppression on the E6 oncoprotein.
Among the three *A. cepa* L. crude extracts, the chloroform extract exhibited the highest inhibition activity against the HPV 16 E6 oncoprotein in viable CaSki cells, followed by its methanol extract and then petroleum ether extract. The chloroform extract of *A. cepa* L. had demonstrated E6 suppression activity from 1 µg/ml onwards and methanol extract exerted E6 inhibition activity from concentrations 12.5 µg/ml onwards.

As illustrated in Figure 4.11, it was observed that the intensity of reddish-brown stains in viable CaSki cells treated with petroleum ether extract had remained the same although the cells were treated with different concentrations of extract. This suggested that the E6 suppression level was the same for all concentrations of petroleum ether extracts. However, the petroleum ether extract showed E6 inhibition effects even at the lowest concentration of extract (1 µg/ml).

In general, the E6 oncoprotein was detected at the cytoplasmic region of the viable CaSki cells. Overall, at all concentrations of all extracts, the CaSki cells were observed to be intact, indicating that no cell lysis had occurred.
Figure 4.11 Staining intensity of CaSki cells treated with *A. cepa* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

*Allium fistulosum* L.

Based on the ICC results shown in Figure 4.12, crude extracts of *A. fistulosum* L. demonstrated a positive suppression trend whereby the reddish-brown stain intensity decreased with the increasing concentrations of crude extracts used. Among the three crude extracts, the petroleum ether extract of *A. fistulosum* L. stood out to be best at suppressing the expression of E6 oncoprotein in the viable CaSki cells when compared to its chloroform and methanol counterparts.

Intensity of the reddish-brown stain indicates a strong suppression effect of the petroleum ether extract against the HPV 16 E6 oncoprotein even at a concentration as low as 1 µg/ml. The petroleum ether extract of *A. fistulosum* L. was observed to
demonstrate same E6 suppression level at different concentrations of extract. At all concentration of extracts, it was observed that only mild reddish-brown stain was present in the viable CaSki cells. This indicates that petroleum ether extract was very effective in suppressing E6 expression.

The chloroform extract inhibited E6 oncoprotein expression at concentration of extract as low as 1 µg/ml while its counterpart, methanol extract only demonstrated E6 suppression at 12.5 µg/ml. Treatment with 12.5 µg/ml of methanol extract left only mild amounts of E6 oncoprotein still present in the viable CaSki cells whereas chloroform extract showed weaker suppression of HPV 16 E6 oncoprotein at the same concentration.

CaSki cells treated with methanol extracts at concentrations 12.5 µg/ml and more exhibited mild staining, indicating strong E6 suppression by the methanol extracts at those concentrations. However, very strong reddish-brown stain was observed at the lowest concentration of methanol extract of 1 µg/ml denoting weak E6 inhibition activity.

Generally, mild lysis was observed among the CaSki cells treated with petroleum ether and methanol extracts of A. fistulosum L. whereas chloroform extracts had a more negative effect on the integrity of the CaSki cells causing a more severe lysis.
Figure 4.12 Staining intensity of CaSki cells treated with *A. fistulosum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

*Allium porrum* L.

The crude extracts of *A. porrum* L. exhibited positive anti-HPV 16 E6 activities when tested against the CaSki cell line. Among the crude extracts of *A. porrum* L., the petroleum ether extract was found to be most successful in suppressing the E6 oncoprotein expression in the viable CaSki cells (Figure 4.13).

At the concentration 1 µg/ml, the E6 expression was classified as mild reddish-brown stain. However, cells treated with 25 µg/ml petroleum ether *A. porrum* L. extract exhibited moderate reddish brown stain indicating moderate suppression by the extract. Nonetheless, E6 suppression activity became strong again as denoted by the presence of
mild reddish-brown stain in CaSki cells treated with petroleum ether extract 50 µg/ml and above.

As for its chloroform extract, it has demonstrated a smooth suppressing trend in decreasing the reddish-brown stain intensity with the increment of concentration of extracts treated against the CaSki cells. Cells treated with chloroform extract at 1 µg/ml exhibited strong reddish-brown stain indicating weak E6 inhibition which gradually increased with increasing concentration of the chloroform extract.

CaSki cells treated with 12.5 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml and 150 µg/ml chloroform extracts of A. porrum L. exhibited moderate staining denoting moderate E6 suppression level. Treatment with chloroform extract 200 µg/ml left only mild amounts of the oncoprotein still present in the viable CaSki cells indicating strong E6 inhibition effects by the chloroform extract at that concentration.

In contrast, the methanol extract of A. porrum L. not only did not suppress the E6 expression in the CaSki cells but surprisingly seen to increase the E6 expression with the increasing concentration of extracts. Cells treated with methanol extract exhibited moderate reddish-brown stain at concentration as low as 1 µg/ml indicating moderate E6 suppression effects of the extract. CaSki cells treated with methanol extract 100 µg/ml and above exhibited strong staining denoting weak E6 suppression activity by the extract.
In general, CaSki cells treated with petroleum ether and methanol extracts of *A. porrum* L. remained morphologically intact while mild cell lysis was seen among cells treated with chloroform extracts at concentrations 150 µg/ml and below. Otherwise, cells remained morphologically intact.

![Figure 4.13 Staining intensity of CaSki cells treated with *A. porrum* L.](image)

Figure 4.13 Staining intensity of CaSki cells treated with *A. porrum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

*Allium sativum* L.

The E6 expression levels in the Caski cells that were treated with the crude extracts of *A. sativum* L. are presented in Figure 4.14. Overall results indicated that the chloroform extract was more suppressive against HPV 16 E6 oncoprotein in CaSki cells as compared to its petroleum ether and methanol counterparts. The chloroform extract
significantly inhibited the HPV 16 E6 at 1 µg/ml whereas the methanol extract strongly suppressed the E6 expression at concentration 12.5 µg/ml. The petroleum ether extracts weakly suppressed the oncoprotein at concentration 25 µg/ml.

At all concentrations of chloroform extracts, only mild staining was seen in viable CaSki cells, indicating very effective E6 inhibition activity by the extract. Cells treated with methanol extracts concentration 12.5 µg/ml and above exhibited mild reddish-brown stains indicating strong E6 suppression by the extract. At the lowest concentration of 1 µg/ml, strong reddish-brown stain was observed denoting weak E6 suppression by the methanol extract.

As illustrated in Figure 4.14, the intensity of reddish-brown stains in CaSki cells treated with petroleum ether extract derived from A. sativum L. decreased with the increasing concentrations of the plant extracts. E6 inhibition was very weakly demonstrated by petroleum ether extracts at both concentrations of 1 µg/ml and 12.5 µg/ml.

Results indicated that strong reddish-brown stain was present in cells treated with 25 µg/ml of petroleum ether extract, denoting weak E6 suppression activity by the extract. Cells treated with 50 µg/ml petroleum ether extract showed only mild amounts of the oncoprotein still present in the viable CaSki cells.

Cells treated with petroleum ether extract of A. sativum L. remained intact while the integrity of CaSki cells treated with chloroform extract were negatively affected where the cells lyzed at concentrations 25 µg/ml and below. Methanol extract of A. sativum L.
caused the cells to lyse at concentrations 50 µg/ml and below. Otherwise, cells remained intact.

![Graph showing staining intensity of CaSki cells treated with different extracts. The x-axis represents the crude extract types (Petroleum ether, Chloroform, Methanol), and the y-axis represents staining intensity (+). The graph shows that methanol extracts produced the strongest E6 suppression followed by chloroform extracts. The crude petroleum ether extract produced the poorest E6 suppression activity.](image)

**Figure 4.14** Staining intensity of CaSki cells treated with *A. sativum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+).

*Allium ursinum* L.

As illustrated in Figure 4.15, the crude extracts of *A. ursinum* L. showed positive anti-HPV type 16 E6 activities. Among the three, methanol extracts produced the strongest E6 suppression followed by the chloroform extracts. The crude petroleum ether extract produced the poorest E6 suppression activity.
The methanol extract demonstrated higher inhibition activity against HPV 16 E6 oncoprotein at concentration 12.5 µg/ml whereas petroleum ether and chloroform extracts showed weaker suppression of HPV 16 E6 at the same concentration. At concentration of 200 µg/ml methanol extract, viable CaSki cells exhibited mild staining indicating strong suppression by the extract.

Treatment of CaSki cells with 1 µg/ml of chloroform extract left very strong reddish-brown stains indicating very weak suppression of E6 by the extract. Gradually, the inhibition activity of HPV 16 E6 was moderately suppressed at concentrations 25 µg/ml and above as denoted by moderate stains observed in the viable CaSki cells.

As denoted by the reduction of reddish-brown stain in CaSki cells, the inhibition activities of HPV 16 E6 oncoprotein by A. ursinum L. petroleum ether extracts increased with the increasing concentrations of the extracts. Cells treated with petroleum ether extracts exhibited very strong stains at 1 µg/ml indicating very weak suppression activity. E6 expression in CaSki cells was further reduced to moderate reddish-brown stains at concentrations 100 µg/ml onwards indicating moderate E6 inhibition activity.

CaSki cells that were treated with crude petroleum ether and chloroform extracts showed no signs of lysis; the cells were intact but mild cell lysis were observed among the cells treated with methanol extract at concentrations 50 µg/ml and 200 µg/ml.
**Figure 4.15** Staining intensity of CaSki cells treated with *A. ursinum* L.. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

*Allium tuberosum* Rottl. (Bunga Kuchai)

A reduction of reddish-brown stain intensity was observed in CaSki cells treated with the increasing concentrations of *A. tuberosum* Rottl. (bunga kuchai) crude extracts as illustrated in Figure 4.16. The reduction in the stain intensity reflects the positive anti-HPV type 16 E6 activities of *A. tuberosum* Rottl. (bunga kuchai) crude extracts. Among the crude extracts, the methanol extracts stood out to be the most effective in inhibiting the HPV 16 E6 expression in the CaSki cells, followed by petroleum ether extract and then chloroform extract.
Mild reddish-brown stain was exhibited in CaSki cells treated with 1 µg/ml of methanol extract indicating strong E6 suppression activity of the extract. The same suppression effect was seen in cells treated with higher concentrations of the extract. In other words, the methanol extract of *A. tuberosum* Rottl. (bunga kuchai) was effective in suppressing the E6 oncoprotein expression even at concentration of as low as 1 µg/ml.

Likewise, the crude petroleum ether extracts also demonstrated a suppressing trend with the increasing concentration of extracts. At 1.0 µg/ml, the reddish-brown stain was moderate but was further reduced to mild stain when 12.5 µg/ml of petroleum ether extract was treated onto the CaSki cells.

The crude chloroform extracts exerted the poorest E6 suppression activity. At 1 µg/ml of extract, the reddish-brown stain was moderate indicating moderate E6 inhibition and was reduced to mild stain at 150 µg/ml of extract, indicating strong E6 suppression at that concentration and the same suppression effect was seen in cells treated with 200 µg/ml of extract.

In general, mild cell lysis was observed among the cells treated with the crude extracts of *A. tuberosum* Rottl. (bunga kuchai). Cells treated with petroleum ether extracts showed lysis at concentrations 25 µg/ml and below. All CaSki cells treated with chloroform *A. tuberosum* Rottl. (bunga kuchai) extract of concentrations 150 µg/ml and below showed mild lysis.
The integrity of CaSki cells treated with methanol extracts were negatively affected where lysis was seen at concentrations 25 µg/ml and above. Otherwise, cells remained morphologically intact.

![Figure 4.16 Staining intensity of CaSki cells treated with \(A.\) *tuberosum* Rottl. (bunga kuchai). Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)](image)

**Allium tuberosum** Rottl. (Daun Kuchai)

As seen in the ICC staining results illustrated in Figure 4.17, petroleum ether extract of \(A.\) *tuberosum* Rottl. (daun kuchai) was found to be most effective in suppressing the expression of HPV 16 E6 oncoprotein as compared to its chloroform and methanol counterparts. CaSki cells treated with 1.0 µg/ml petroleum ether extract exhibited mild
reddish-brown stains indicating very strong E6 suppression by the extract. This suppression effect remained the same at all concentrations of petroleum ether extracts.

Based on the staining results, both chloroform and methanol extracts demonstrated similar E6 suppression activity. Cells treated with *A. tuberosum* Rottl. (daun kuchai) chloroform and methanol extracts exhibited moderate reddish-brown stains indicating moderate HPV 16 E6 oncoprotein inhibition activity at concentration as low as 1 µg/ml. When concentration of extracts were increased to 100 µg/ml and above, both chloroform and methanol extracts exerted strong inhibition against HPV 16 E6 oncoprotein which was reflected by mild reddish-brown stains observed in the viable CaSki cells.

CaSki cells treated with petroleum ether and methanol extracts of *A. tuberosum* Rottl. (daun kuchai) remained intact at all concentrations. However, mild lysis was observed among cells treated with chloroform extracts at concentrations 150 µg/ml and below. Otherwise, cells were all intact.
Figure 4.17 Staining intensity of CaSki cells treated with *A. tuberosum* Rottl. (daun kuchai). Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)

4.7.2 Comparison of Anti-HPV Type 16 E6 Activities of Selected *Allium* spp.

A total of 21 crude extracts obtained from seven species of *Allium* were screened for anti-HPV type 16 E6 activities in the present study. Through ICC, different crude extracts from different *Allium* spp. exhibited different range of anti-HPV type 16 E6 activities. In other words, these crude extracts from the *Allium* spp. each possess their own potential in inhibiting the expression of E6 oncoprotein in CaSki cells.

The summaries of E6 expression of CaSki cells treated with the crude extracts of the *Allium* spp were shown in Figures 4.18(a-c). Overall results showed that the crude extracts act in a dose-dependent manner where greater inhibition activity against HPV
16 E6 oncoprotein was observed in higher extract concentration. This was indicated by the reduction in reddish-brown staining intensities with increasing concentrations of extracts. The petroleum ether extracts of the Allium spp. was the better anti-HPV type 16 E6 agents when compared its chloroform and methanol counterparts. Chloroform extracts were moderate while methanol extracts were poor in HPV 16 E6 oncoprotein inhibition.

A total of 20 crude extracts (95.24%) exhibited positive anti-HPV type 16 E6 oncoprotein activity. 16 crude extracts (76.19%) were effective in inhibiting the HPV 16 E6 oncoprotein expression at the lowest concentration of extract of 1 µg/ml. 23.81% of crude extracts showed strong HPV 16 E6 inhibition activity even at the lowest concentration of extract of 1 µg/ml.

Present findings show that A. tuberosum Rottl. (bunga kuchai), stood out to be the most effective plant in inhibiting the HPV 16 E6 oncoprotein followed by A. tuberosum Rottl. (daun kuchai), A. fistulosum L., A. sativum L., A. porrum L., A. cepa L. and A. ursinum L. in a decreasing order. Allium tuberosum Rottl. (bunga kuchai) exhibited significant suppression against the HPV 16 E6 oncoprotein in all its petroleum ether, chloroform and methanol extracts even at the lowest concentration of 1 µg/ml.

**Crude Petroleum Ether Extracts**

As illustrated in Figure 4.18 (a), 28.57% of petroleum ether extracts was found to show strong E6 inhibition activity at all concentrations of extracts. Allium fistulosum L. and A. tuberosum Rottl. (daun kuchai) were the most effective in inhibiting against the HPV
16 E6 oncoprotein activity (Table 4.20 and 4.21). This was indicated by CaSki cells treated with 1 µg/ml extracts from both plants showing mild reddish-brown stains denoting strong E6 inhibition by the extracts.

The intensity of reddish-brown stains in CaSki cells treated with petroleum ether extracts derived from *A. sativum* L. and *A. ursinum* L. decreased with the increasing concentrations of the plant extracts. This indicates that these two plants exhibited anti-HPV 16 E6 oncoprotein activities in a dose-dependent manner where greater inhibition was seen at higher concentration of extracts used.

Weak E6 suppression activity was exhibited by extracts from the *A. cepa* L., *A. sativum* L. and *A. ursinum* L.. At 1 µg/ml, CaSki cells treated with *A. cepa* L. extracts exhibited strong reddish-brown stains indicating weak suppression activity. *Allium sativum* L. and *A. ursinum* demonstrated weaker inhibition activity at the same concentration of extract. CaSki cells treated with concentration of 200 µg/ml of *A. cepa* L., *A. sativum* L. and *A. ursinum* L. had respectively exhibited strong, mild and moderate staining indicating that *A. cepa* L. was weak (Table 4.22), *A. sativum* L. was strong and *A. ursinum* L. was moderate at inhibiting the HPV 16 E6 activity.

It was also observed that for extracts of *A. cepa* L., the E6 suppression activity did not improve with the increasing concentration of extract used but instead remained weak as indicated by the same strong reddish-brown intensity in the cells. The inhibition activities improved at the concentration 50 µg/ml when stains in CaSki cells treated with *A. sativum* L. extract were reduced to mild while reddish-brown stains in cells
treated with *A. ursinum* L. extract remained strong indicating weak suppression by the extract.

**Crude Chloroform Extracts**

Based on the staining results illustrated in Figure 4.18(b), 14.29% of chloroform extracts exhibited HPV 16 E6 inhibition activity at all concentrations of extracts. *Allium sativum* L. was the most effective E6 suppression agent by exhibiting mild reddish-brown stain in CaSki cells indicating strong inhibition activity at all concentrations of extracts (Table 4.23). This was followed by *A. fistulosum* L., *A. tuberosum* Rottl. (daun kuchai) and *A. tuberosum* Rottl. (bunga kuchai) in a decreasing manner.

CaSki cells treated with 1 µg/ml of *A. fistulosum* L. and both variants of *A. tuberosum* Rottl. exhibited moderate reddish-brown stains denoting moderate E6 suppression activities by these extracts. Treatment of 100 µg/ml chloroform extracts of *A. fistulosum* L. and *A. tuberosum* Rottl. (daun kuchai) had left only mild amounts of E6 oncoprotein in the cells indicating strong E6 inhibition by these extracts at this concentration. However, the suppression level remained moderate for *A. tuberosum* Rottl. (bunga kuchai) at the same concentration of extract used.

*Allium ursinum* L. chloroform extract was poorest in showing E6 inhibition effects (Table 4.24). At 1 µg/ml, CaSki cells exhibited strong reddish-brown stains indicating weak suppression activity by the extract. However, HPV 16 E6 oncoprotein was better suppressed at higher extract concentrations of 25 µg/ml onwards.
Crude Methanol Extracts

The resulting ICC staining intensities of the CaSki cells treated with methanol extracts of the *Allium* spp. are shown in Figure 4.18(c). 14.29% of the methanol extracts exhibited HPV 16 E6 inhibition activity at all concentrations of extracts. *Allium tuberosum* Rottl. (bunga kuchai) performed outstandingly in suppressing the E6 oncoprotein expression (Table 4.25). It was observed that methanol extract of *A. tuberosum* Rottl. (bunga kuchai) produced strong E6 inhibition even at the lowest concentration of extract of 1 µg/ml. This was indicated by the mild reddish-brown stain found in CaSki cells treated with the extract and this strong suppression activity was seen at all concentrations of *A. tuberosum* Rottl. (bunga kuchai) methanol extracts.

Surprisingly, the methanol *A. porrum* L. extracts did not inhibit the E6 oncoprotein instead induced the expression of E6 in CaSki cells (Table 4.26). It was observed that cells treated with 1 µg/ml methanol extract of *A. porrum* L exhibited moderate reddish-brown stains indicating moderate E6 suppression activity. When the concentration of *A. porrum* L. methanol extract was increased to 100 µg/ml extract, strong reddish-brown stains appeared in the cells. This phenomenon continued even at the highest concentration of extract 200 µg/ml.
Figure 4.18(a) The summary of reddish-brown stain intensity of CaSki cells treated with crude petroleum ether extracts of selected *Allium* spp. as determined by ICC. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)
Figure 4.18(b) The summary of reddish-brown stain intensity of CaSki cells treated with crude chloroform extracts of selected *Allium* spp. as determined by ICC. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+).
Figure 4.18(c) The summary of reddish-brown stain intensity of CaSki cells treated with crude methanol extracts of selected *Allium* spp. as determined by ICC. Intensity of reddish-brown stain is classified as no stain (0), mild (1+), moderate (2+), strong (3+) and very strong (4+)
Table 4.20 Appearances of CaSki cells treated with crude petroleum ether extracts of *A. fistulosum* L.

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Concentration of Crude Extract (µg/ml)</th>
<th>Reddish-brown Stain Intensity</th>
<th>Other Observations</th>
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<tr>
<td></td>
<td>200</td>
<td>(+)</td>
<td>Mild cell lysis was seen among cells</td>
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<td>1</td>
<td>(+)</td>
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### Table 4.21 Appearances of CaSki cells treated with crude petroleum ether extracts of *A. tuberosum* Rottl. (daun kuchai)

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Concentration of Crude Extract (µg/ml)</th>
<th>Reddish-brown Stain Intensity</th>
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Table 4.22 Appearances of CaSki cells treated with crude petroleum ether extracts of *A. cepa* L.

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Table 4.23 Appearances of CaSki cells treated with crude chloroform extracts of *A. sativum* L.

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<th>Appearance</th>
<th>Concentration of Crude Extract (µg/ml)</th>
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Table 4.24 Appearances of CaSki cells treated with crude chloroform extracts of *A. ursinum* L.

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Table 4.26 Appearances of CaSki cells treated with crude methanol extracts of *A. porrum* L.

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<th>Appearance</th>
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5.1 Preparation of Experimentation Materials

5.1.1 Plant Extraction

Infusion Method

Crude extracts from the *Allium* spp. were obtained using the infusion method, which is a cold extraction method. Grounded plant materials were infused with the preferred solvent at room temperature (27°C) for a period of time, i.e. 72 hours in the present study. Intermittent shaking was optional when the infusion process takes place. When the plant material settled, the upper solvent-extract mixture were decanted off into another container and replaced with fresh solvent. In between changes of solvent, it was crucial to thoroughly dry the samples to prevent the carry-over of previous solvent trace that may interfere with the next series of infusion (Houghton and Raman, 1998).

Infusion method is advantageous because heating is not required during the extraction process. This therefore helps preserve most of the phytochemicals of the plant. However, some novel and important compounds in the plants might not be extracted since there was no heat induction. Application of heat during extraction may help increase extraction or create chemical reactions in order to obtain certain form of derivatives (Houghton and Raman, 1998).

After 72 hours of infusion, the extract was filtered to remove the plant debris before subjected to evaporation. Evaporation was important as it helps concentrate the extract for chemical and biological analysis. However, the rotary evaporation method used in
the present study may have its own share of disadvantages. Firstly, volatile oils may be lost from the extract during evaporation as the extract may suffer from thermal degradation reactions when heat is applied. Secondly, sudden boiling may lead to loss of liquid from the flask as it occurs easily. To avoid this, anti-bumping granules were added into the solvent-extract solution (Houghton and Raman, 1998).

Another disadvantage of this method was the possible contamination by trace residues from other extracts, thus the glass apparatus were kept scrupulously clean all the time. Traces of solvents which may cause interference during analysis will be removed when the extract is concentrated. It was crucial to carry out evaporation at temperatures as low as possible to avoid disintegration of the phytochemical constituents (Houghton and Raman, 1998).

To avoid further loss of extract weight after evaporation, empty bottles where final evaporation will take place were weighed beforehand so that the final yield of extract can be calculated easily without having to transfer the extract into another bottle after evaporation. To ensure complete removal of solvent, extracts were left to dry for a period of time until a constant weight was obtained (Houghton and Raman, 1998). Among the three crude extracts, the chloroform extracts were probably more pure than its counterparts as chloroform is easily vapourized and less effort was needed to remove the solvent after extraction. The concentrated extracts were kept in bottles at 4°C prior to use.
In the present study, the leaf variants of *Allium* spp. (e.g. *A. porrum* L. and both *A. tuberosum* Rottl. variants) were found to have low dry weight values but showed high total yield of crude extract as compared to the bulbous *Allium* variants (e.g. *A. cepa* L., *A. sativum* L. and *A. ursinum* L.). It was observed that the leaf variants had higher yield of each crude extracts when compared to the bulbous variants, hence, increasing their total yield of crude extract. This may be related to the effect of the preparation method towards both the leaf and bulb variants of the *Alliums* in the present study. The possibility of many potential chemical reactions taking place before, during and after the preparation of the plant materials may potentially contribute to the change of the chemical contents of the *Allium* samples.

For example, the chemical constituents of intact *A. sativum* L., do not yield allicin but when crushed, cut or chopped, allicin will be released upon enzymatic reactions catalyzed by allinase. The unstable allicin will easily be converted into other organosulfur compounds (Lancaster and Shaw, 1989; Block, 1985). If garlic oil was prepared using steam distillation, it will yield allyl, diallyl and dimethyl sulfide compounds (Banerjee *et al.*, 2003; Kritchevsky, 1991).

Likewise, the presence of organosulfur compounds in onion or *A. cepa* L. depends on the variety of preparation conditions. For example, when extracted using steam, fresh onion matter will produce propionaldehyde and dipropyl disulfide while infusion with ethyl alcohol at temperature below 0°C, the lachrymatory precursor will be extracted (Yang *et al.*, 2004).
Solvents

In this infusion method, the types of solvents used play an important role in ensuring the maximum extraction of the plant’s phytochemicals. There is a general principle where ‘like dissolves like’ thus non-polar solvents will extract non-polar substances while polar substances will be extracted by polar solvents. In the present study, petroleum ether, chloroform and methanol were used for the sequential extraction with the purpose of obtaining a wider range of phytochemicals from the *Allium* plant materials.

Petroleum ether has very similar extraction properties to hexane thus it dissolves wax compounds, fats, fixed oils and volatile oils. Chloroform is adopted to extract alkaloids, aglycones and volatile oils while methanol extracts sugars, amino acids and glycosides from the samples. In between changes of solvent, it was crucial to thoroughly dry the samples to prevent the carry-over of previous solvent trace that may interfere with the next series of infusion (Houghton and Raman, 1998).

As we know, some novel compounds of the *Allium* spp. were lipid- and water-soluble and these compounds may be activated in either the hydrophobic or hydrophilic layers of the cell. However, we may not deny that some of these compounds or its derivatives are amphiphilic. Amphiphilic compounds have both hydrophobic and hydrophilic structural regions which allow them to dissolve in both water and to some extent in non-polar organic solvents. Chloroform is considered to have intermediate polarity among the three solvents used in this study whereby it has a bigger dielectric constant (4.8) than petroleum ether (2.28) but less polar than methanol (33) (Solomons, 1996; Lide, 1993). Thus, the amphiphilic nature of the organosulfur compounds may explain the
probable increase of bioactive effects of crude chloroform extracts in the present study, if any.

Extraction using chloroform may introduce new derivatives because the solvent may cause weak chemical reactions in order to help solubilize the solute. These new derivatives were probably responsible for the anti-tumour activity against HCT-116. The primary sulfur-containing constituents were S-alk(en)yl-L-cysteine sulfoxides (ACSO) and γ-glutamylcysteines which would be converted into other compounds through different metabolic pathways such as hydrolysis, oxidation, condensation and disintegration into more beneficial compounds such as DADS, DATS, SAC, ajoene and dithiins (Corzo-Martinez et al., 2007).

In the present study, the lipid-soluble compounds of the *Allium* plants such as SAC, SAMC and alliin were assumed to be extracted by petroleum ether while water-soluble compounds such as allylmercaptan, allyl and diallyl sulfides may be extracted by methanol. Phenolic compounds may be extracted into petroleum ether, chloroform or methanol, depending on their structure configuration (Houghton an Raman, 1998). Quercetin, a phenolic compound found abundantly in onion (Corzo-Martinez et al., 2007; Amagase et al., 2001) may be extracted into the crude chloroform extract of *A. cepa* L., contributing to this plant being the *Allium* sample with highest total phenolic content (i.e. 820±0.00 mg GAEs/100 g sample).

Several studies have shown that both lipid- and water-soluble organosulfur compounds from the *Allium* plants contributed to their anti-tumourigenic activities. According to
Sigounas et al. (1997), carcinogenesis can be inhibited by DAS, DADS, DATS and SAC found in *A. sativum* L. while ajoene, SAMC and methiin provide for anti-proliferation activities of garlic and onion. These organosulfur compounds were reported to induce apoptosis in human cell cultures (Dirsch et al., 1998; Sakamoto et al., 1997; Sigounas et al., 1997).

In addition to that, the antioxidant activities of the *Allium* plants in the present study were claimed to be provided by the phenolic compounds (e.g. flavonoids), saponins, lectin, selenium, ajoene, DAS, DADS, DATS, SAC and SAMC, which were present in the crude extracts of these plants (Dimitrios, 2006; Amagase et al., 2001; Borek, 2001; Imai et al., 1994). Another study had reported that that extracts with highest content of allicin and other thiosulfinates such as ajoene, DADS and DATS showed higher antiviral activities and extracts that were virucidal were also cytotoxic towards HeLa and Vero cells (Weber et al., 1992).

**Crude Extract Storage**

As it was vital that all medium, extracts and solutions used were sterile as contamination may affect the growth of cells subjected for test system, the extracts were made sure that they were sterile before use. A water-miscible solvent such as 100% DMSO was used to dilute the plant extracts as it does not comply with bacterial growth. DMSO is suitable for compounds that are not soluble in aqueous solution and at concentration below 3% v/v; DMSO does not cause toxicity to cells (Houghton and Raman, 1998; Wilson, 1992).
5.1.2 Cell Cultivation and Maintenance

Handling of Glassware and Materials

As sterility is an important criterion in cell culture, handling of all glassware and materials during cell culture were carried out under sterile conditions with the practice of aseptic techniques to prevent any form of contamination done. Thus the usage of sterile glassware, culture media and solutions are essential (Houghton and Raman, 1998). Glassware and other necessary materials needed for cell culture were soaked in non-cell toxic detergent and rinsed properly before being autoclaved and all items were taped with an indicator tape. Lids on all bottles were loosened to allow proper aeration when being autoclaved and immediately tighten before removing from the autoclave. Pipettes were dry-sterilized using high temperature of 180°C in canisters.

Contamination

The common forms of contamination are bacterial and fungal contamination. These are usually airborne and arise from poor aseptic techniques and improper sterilization and storage of materials and reagents. Contamination is usually detected based on visual observation such as cloudy media showing bacterial contamination and the presence of hyphae indicating fungal contamination. Attempts to cure or resuscitate contaminated cells using excessive amounts of antibiotics, anti-fungal or anti-viral agents will do no good but instead harm the cells (Martin, 1994).

If there should be any form of contamination, once it was identified was not subtle but must be gotten rid of as soon as possible. To rid the contamination, all contaminated cell culture flasks were discarded and all glassware, media and solutions used were re-...
sterilized. The entire working surface and incubator exposed to contaminated cultures were cleaned.

**Cell Culture Medium**

In addition to maintaining the sterility of the apparatuses and working environment, the right usage of medium for cell culture also affects the efficiency in breeding the cells. To ensure that, medium used in cell culture must at least contain one buffer and is isosmotic to the cells. Thus, it is important to make sure that these medium used in cell culture remained at pH 7.4 because pH level below or above this value is toxic to the cells (Martin, 1994) and inhibits cell growth (Griffiths, 1992). Furthermore, the medium must have within itself nutrients such as vitamins, amino acids and glucose as an energy source for the cells’ healthy growth. This is important to ensure cell metabolism occur at a normal level. For cells to be able to grow; there must also be a supply of growth factors, lipids and hormones, which were most commonly provided by addition of a serum (Martin, 1994).

**Bathing Fluids**

Nevertheless, it is also crucial that the bathing fluid used is capable of maintaining the correct pH and this is secured by adding a suitable buffer. Most often the fluid that bathes the cells has the same concentration as of the solute molecules inside the cells. Normal cell survival also requires bathing solution that is little acidic and compatible with the cell. The production of CO$_2$ by the cells made bicarbonate buffer a more preferable buffer than the non-bicarbonate ones. Although non-bicarbonate buffers such as HEPES have been successfully used in many cell systems and had allowed a more
strict control of the system’s pH, nonetheless, these buffers may be toxic to the cells (Martin, 1994).

**Trypsinization**

During cell culture maintenance, the usage of a proteolytic enzyme is needed to remove the cells from the surface of the culture flask as cells have glycoprotein, extra cellular proteins and proteoglycans that allow the attachment of the cells to the surface of those specially pretreated culture flasks. In this study, the trypsin enzyme was used. In the present study, trypsin was stored at -20°C prior to use and was thawed at the same temperature as that was used for cultures (37°C) before use. This was important to avoid shocks induced by wide changes in the cell cultures’ temperature. Incubation of cells with trypsin should not take too long (i.e. not more than five minutes) as the enzyme will cleave the cell membrane proteins, resulting in damage to the cells (Martin, 1994). Serum was added after trypsinization to arrest proteolysis (Maurer, 1992).

**5.2 Cytotoxicity Screening Using NR Assay**

In the present study, the cytotoxic activity of *Allium* spp. against MRC-5, CaSki and HCT-116 was evaluated. The NR assay was used to investigate the cytotoxicity capacity of the *Allium* spp. crude extracts. The NR assay is one of the most sensitive cytotoxicity assays in detecting early toxicity by revealing statistically significant results between the controls and the treated cells (Fotakis and Timbrell, 2006). It is a simple and rapid assay which can be conveniently carried out using the 96-well microtitre plates (Borenfreund and Puerner, 1986).
The NR assay was performed after the MRC-5, CaSki and HCT-116 cells were incubated with *Allium* crude extracts for 72 hours. The 72 hours test period was recommended over the 24 hours test to avoid false negative indications of cytotoxic activity (Riddell *et al.*, 1986). This was because some bioactive compounds, particularly those that inhibit cell proliferation may need longer time to exert their cytotoxicity. In addition to that, the NR assay is dependent on the growing number of the cells thus a shorter incubation period will result in failure to discriminate the potential cytotoxic activity of the chemicals.

Borenfreund and Puerner (1985) suggested pre-incubation of the NR medium overnight prior to use at 37°C to allow the precipitation of dye crystals. This was because the presence of dye crystals in the cell culture may interfere with the results of the assay as it may create false results. In the present study, NR medium was firstly pre-incubated overnight at 37°C and then centrifuged for 10 minutes at 1,000 rpm twice right before use to facilitate the removal of the dye crystals.

It was also researched that monolayer cell cultures that are confluent are less sensitive against testing agents. Therefore, it was recommended that cell cultures used for NR assay should only have 60% to 70% confluence when subjected to test to ensure maximum exposure of the cells towards the testing agent (Borenfreund and Puerner, 1986).

As soon as the NR incubation period was over, the cells were rapidly rinsed with a washing solution to remove extracellular NR and also to prevent the detachment of the
cells when subjected to the subsequent procedure of the assay. The washing solution should not be left too long on the cell cultures, briefly 1 to 2 minutes was sufficient. It was important to ensure that the washing period does not take too long as it will lead to the pre-mature extraction of NR dye from the viable cells resulting in false results. Expired or contaminated solution may not be as effective in extracting the dye thus showing low colour intensity when reading the microtitre plate at 540nm. Low colour intensity denotes low cell viability (Borenfreund and Puerner, 1985; 1986).

5.3 Cytotoxic Activities of Selected *Allium* spp.

For the cytotoxicity screening in the present study, whole or crude extracts from the selected *Allium* plants were tested against human tumour cell lines. It was claimed by Liu, (2003) that the synergistic effects of the phytochemicals in whole foods added to their potential as bioactive compound thus exerting more health benefiting effects than an isolated compound alone. However, in the present study, the *Alliums* were not active in inhibiting the growth of human tumour cells (i.e. MRC-5, CaSki and HCT-116 cells).

Overall results showed that the percentage of inhibitions by the crude *Allium* extracts against MRC-5 did not exceed 50% at concentration of extracts of a 100 μg/ml and below. Therefore, no IC$_{50}$ was registered thus reflecting that the *Allium* extracts were not active against MRC-5. However, the petroleum ether extracts of *A. cepa* L., *A. sativum* L. and *A. ursinum* L. and chloroform extract of *A. porrum* L. exhibited 50% of cell growth inhibition when tested against the CaSki and HCT-116 cells respectively. These extracts were cytotoxic against the two cell lines but they were considered not active (Chen *et al*., 1988; Geran *et al*., 1972).
Although very little literature data were available about the cytotoxic effects of whole *Allium* plants’ extracts, it is possible to relate why the *Allium* extracts in the present study were not active cytotoxic agents. Numerous researches had evidently showed the use of specific compounds isolated and prepared through different procedures from *Allium* plants as effective cytotoxic agents against human tumour cells. It was also important to acknowledge that specific *Allium* compounds with high purity levels enhanced their effectiveness as cytotoxic agents against human tumour cells.

The 84.3% pure lipid-soluble DADS was reported in a study to cause apoptosis which lead to the growth inhibition of human breast cancer cell lines KPL-1, MCF-7, MKL-F and MDA-MB231 at an IC\(_{50}\) of 1.8-18.1 µM (Nakagawa *et al*., 2001). In Li *et al.* (2002), the *A. sativum* L. cyclohexane extract was isolated and purified using a series of chromatography to produce an active compound, Z-ajoene. This compound was effective in inhibiting the growth of HCT cells and gave an IC\(_{50}\) of 19.6 µM. This compound was reported to have purity level of more than 98.0%.

Some studies demonstrated that different *Allium* extracts work via different inhibition mechanism(s) and may be detected using cytotoxic screening systems other than the NR assay. Seki *et al.* (2000) showed that steam-distilled garlic and onion oils suppressed the proliferation of the HL-60 cells in a dose-dependent manner using the LDH assay. The anti-proliferation activities exhibited by both garlic and onion oils were reported to be comparable to DMSO. However, the HL-60 growth inhibition was not due to cytotoxic activities of garlic and onion oil. Instead, it was the ability of these *Allium* oils to induce differentiation of the HL-60 cells into granulocytic lineage that has caused the
suppression of its cells’ growth. The HL-60 cells’ differentiation was assayed by nitroblue tetrazolium (NBT) reduction (Seki et al., 2000).

Ajoene, a compound of crushed garlic, was found to induce HL-60 cells’ differentiation by a mechanism differs from that of garlic oil by inducing apoptosis in HL-60 cells via stimulation of peroxide production and activation of NF-κB (Dirsch et al., 1998). In an unrelated study, aqueous garlic extracts were better in inhibiting the growth of HepG2 cells as compared to the commercially available lipid-soluble isolated compound, DADS. The aqueous extracts were more effective than DADS in inducing cell cycle arrest and apoptosis in the hepatocarcinoma cells. The effect of cell growth inhibition was observed to be prolonged using the aqueous extracts than that of DADS (Martino et al., 2006).

In a recent study, aqueous extracts of Allium vegetables (i.e. leek, yellow onion, green onion and garlic) showed effective cell growth inhibition activity when tested against eight different human tumour cell lines. The aqueous garlic extract was found to be most potent with the highest content of antioxidants. It was suggested that the synergy of anti-proliferative and antioxidant activities of the Allium plants was contributing to its chemopreventive potential. The anti-proliferative and antioxidant activities of the Allium plants were measured by NBT reduction and ORAC assay (Boivin et al., 2009).

Another observation in the present study had revealed that the less polar solvent extracts of Allium (e.g. petroleum ether and choloroform extracts) were responsible for the cytotoxic effect against the human tumour cells (i.e. CaSki and HCT-115). Indirectly,
this had shown that it was the less polar constituents of *Allium* (e.g. lipid-soluble compounds) that were playing the role in inhibiting the tumour cells’ growth.

Numerous studies have reported on the anti-tumour effects of lipid-soluble organosulfur compounds of *Allium*. For instance, Knowles and Milner, (1998) reported that DADS, was cytotoxic against HCT-15, a cell line similar to HCT-116. DADS exerted its effect by suppressing p34cdc2 kinase activity and inducing cell cycle arrest in HCT-15. Z-ajoene was reported to inhibit HeLa cells’ growth with IC$_{50}$ of 17.9 µM and HeLa cells are human cervical cancer-derived cells (Li *et al*., 2002).

The cell growth inhibition activity of the organosulfur compounds were said to be depended on the number of present allyl- and/or sulfur groups present whereby the more the group in a compound; the more effective it will be in suppressing cell proliferation (Sundaram and Milner, 1993). For example, the application of 100 µmol/L DADS was able to perform 100% cell growth inhibition on HCT-15 but 200 µmol/L of S-allylmercaptocysteine (SAMC) was needed to exert the same activity (Knowles and Milner, 1998).

Another sulfur compound, Z-ajoene contained three sulfur atoms while allicin only have two sulfur atoms and Scharfenberg *et al*., (1990) reported that the cytotoxic effect of Z-ajoene was twice more active than allicin. In Li *et al*., (2002) Z-ajoene demonstrated better cytotoxic effects than allicin. Thus, it is reasonable to believe that not every organosulfur compounds in *Allium* would exert equal cell growth inhibitory activity.
The difference of *Allium* cytotoxicity against CaSki and HCT-116 cells may be attributed to the different molecular and cellular structure as well as the malignant capacities and proliferative growth of these cells. CaSki is human cervical cancer-derived cell line whereas HCT-116 is human colon carcinoma-derived cell line. CaSki cells are HPV type 16 positive and produce endothelin-1 (ET-1) which induces cell growth response and expresses mRNA for endothelin A receptor (ET₄₅AR) in CaSki (Venuti *et al.*, 2000). The alteration of physiological function and up-regulation of cell growth factors may result in an unbalanced cell growth.

It was speculated that ET-1 played an important role in the regulation and promotion of CaSki cells’ growth (Battistini *et al.*, 1993). Therefore, the presence of compounds that antagonize the action of ET-1 would directly affect the growth of the CaSki cells. Based on the results in the present study, CaSki cells were most susceptible towards the effects of *Allium* crude extracts. It is reasonable to suggest that the *Allium* extracts may possess ET-1 antagonist effects which results in inhibition of CaSki cells’ growth.

On the other hand, HCT-116 cells are P-glycoprotein (P-gp) under-expressing cells (Shionoya *et al.*, 2003). P-gp is an efflux pump that facilitates the removal of anti-tumour agents from the cells (Takara *et al.*, 2002). An increased expression of P-gp helps reduce the absorption of drugs that are substrates of P-gp (e.g. Ca²⁺ antagonists, diosgenin, doxorubicin) (Akiyama, 2002; Varadi *et al.*, 2002). In this study, it was observed that the cytotoxic activity against HCT-116 of the chloroform extract of *A. porrum* L. was greater than other *Allium* extracts and registered an IC₅₀. This may be due to the presence of some chemical constituents in the chloroform extract of *A.*
porrum L. that also inhibit the P-gp expression in the HCT-116 cells compared with the rest of the Allium extracts.

Previous studies have also related the anti-tumour activity of Allium organosulfur compounds to the induction of apoptosis. It was believed that the exposure of the cancer cells to DADS and DATS extracted from A. sativum L. induce apoptosis and cell cycle alteration. This was supported by the evidence of significant G2/M phase arrest in human lung carcinoma cells and G1/S arrest in gastric cancer cells after treatment with the organosulfur compounds (Wu et al., 2005; Yuan et al., 2004; Li and Lu, 2002). An isolated compound from A. tuberosum Rottl., thiosulfinates, was reported to induce apoptosis via the caspase-dependent and -independent pathways in PC-3 (Kim et al., 2008).

Karasaki et al., (2001) evaluated the anti-cancer and chemopreventive potentials of lectin from A. sativum L. and found that the garlic lectin effectively inhibited the growth of human tumour cell lines (U937 and HL-60) without harming or damaging the normal cells or tissues. This observation suggested that the garlic lectin may be a selective anti-tumour agent and this compound was suspected to exert its anti-cancer properties by binding to the cell membrane of the tumour cells. In the same study, lectin was also found to have pronounced anti-HIV activity. In view that CaSki was a cervical cancer-derived cell line (American Type Culture Collection, 2004) and cervical cancer is caused by high-risk HPVs (i.e. HPV type 16), thus it can be proposed that garlic lectin may have caused the same inhibition against the CaSki cells’ growth.
Hence, it is important that the efficacy and anti-tumour effects of *Allium* vegetables are further exploited to warrant better understanding of their anti-tumour mechanisms(s) in cancer chemoprevention.

### 5.4 Antioxidant for Cancer Prevention

For the past few decades, researchers have shown more interest in the role of dietary antioxidants in preventing various diseases in human such as cancer, cardiovascular diseases, diabetes and neurodegeneration. It was discovered that the inverse relationship between the intake of dietary antioxidants and cancer has helped strengthened the association of vegetables and fruits consumption with the reduced rate of cancer incidence (Greenwald, 1996; Potter and Steinmetz, 1996; Wattenberg, 1992; Wattenberg, 1985).

Many of the antioxidants compounds were naturally occurring in plants and they had gained the recognition as free radical and/or reactive oxygen species scavengers. Numerous researches have reported how free radicals, oxidants with an iron-sulfur center and other oxygen reactive species could cause oxidative damages to macromolecules (i.e. DNA, lipid, protein and amino acid). Both cell injury and cell death may occur resulting in carcinogenesis. Thus, by preventing free radical attack on these biomolecules, many biochemical and molecular alterations could be avoided and tumour initiation and promotion may be inhibited. Although antioxidants were broadly defined as compounds that hinder oxidative damages of the cellular macromolecules, many antioxidants were specific and offer a single protection mechanism against oxidative damages.
For instance, some antioxidants may scavenge free radicals, quench or react with free radicals thus sparing the targets while some may transform the reactive species into non-reactive products. Other antioxidants may chelate the metal ions that catalyse the free radical chain reaction. Therefore, the antioxidative potential of an extract may vary in different antioxidant bioassay system and this is not uncommon as an effective antioxidant in one assay system is not necessarily an effective antioxidant in another (Miliauskas et al., 2004).

In the present study, crude extracts of selected *Allium* spp. were investigated for their antioxidative properties using the FTC assay, the DPPH radical scavenging assay, the reducing power assay and the β-carotene bleaching inhibition assay. Total phenolic and flavonoid contents were determined using the Folin-Ciocalteau reagent and the AlCl₃ colorimetric assay. These evaluations were important to establish the potential antioxidant activities of the *Allium* crude extracts as effective antioxidant compounds in line with the first, second and third line antioxidant defence. However, no comprehensive literature data on antioxidant activities of these *Alliums* were available for comparison.

**5.4.1 Inhibition of Initial Oxidation as First Line Antioxidant Defence**

The potential of plant extracts as preventive antioxidants in inhibiting the formation of free radicals can be investigated using the autoxidation of linoleic acid in water-alcohol system or FTC assay and the TBA assay (Vimala, 2008). The FTC assay measures the initial formation of free radicals while TBA assay evaluates the secondary oxidation of
lipids. In most cases, LPO is initiated by the presence of free radicals such as peroxyl radicals.

Peroxyl radicals were formed during LPO chain reactions when any ROS species loses one of its electrons and aggressively seeks an electron to stabilize itself again. These species would seek hydrogen atom from polyunsaturated fatty acid (PUFA) side chain (i.e. linoleic acid, arachidonic acid) in membrane lipids. The formation of peroxyl radicals is the major chain-propagating step in lipid peroxidation and if not stopped, will create more damages to the body in long term (Helen et al., 2000). Therefore, the first line of antioxidant defence works by preventing the first chain initiation by scavenging free radicals in the system.

In the present study, the FTC assay was used to evaluate the potential of the *Allium* plants in playing the role as the first line of antioxidant defence to inhibit the initiation of LPO by free radicals. This was because the thiocyanate ion binds specifically to Fe$^{3+}$ only and forms the Fe$^{3+}$/thiocyanate reddish-brown complex which is easily detected at 500 nm (Wong and Kitts, 2001). Jayaprakasha et al., (2001) demonstrated that the oxidation of linoleic acid is retarded when antioxidants are present.

BHA was used as standard positive reference in this assay. On day eight, the absorbance of the negative control sample reached its maximum and the values obtained were taken as a 100% lipid peroxidation. Based on that, the percentage of antioxidant activity for the crude extracts of the *Allium* spp. was calculated (Emami et al., 2007).
Based on the results, no *Allium* crude extract was observed with absorbance values greater than the negative control at the end point (day eight) thus suggesting positive antioxidative potential of the *Allium* plants in the present study. Overall, 57.14% of the chloroform extracts gave antioxidant activities that were as good as BHA when compared to its petroleum ether and methanol extracts while 28.57% of the petroleum ether extracts were similar to BHA. No methanol *Allium* extracts exhibited antioxidant activity better than that of BHA.

Overall results showed that the petroleum ether and chloroform extracts of the leaf *Allium* spp. (i.e. *A. fistulosum* L., *A. porrum* L., and two variants of *A. tuberosum* Rottl.) exhibited more promising antioxidant activities when compared to its bulbous counterparts (i.e. *A. cepa* L., *A. sativum* L. and *A. ursinum* L.). However, no such clear trend can be seen with methanol *Allium* extracts as both bulbous and leaf *Allium* spp. exhibited poor antioxidant activities.

This was consistent with Erkan *et al.*, (2008) that had argued that hydrophobicity and solubility of a plant extracts may affect the potential of an antioxidant in the FTC assay. As such, it was summarized that the petroleum ether and chloroform extracts of *Allium* plants in the present study are effective antioxidants in the first line of antioxidant defence. These plants prevented the formation of lipid peroxides thus inhibiting the initiation of a free radical chain.

Other evidents showed that *A. cepa* L. oil had effectively inhibited nicotine-induced LPO in rats when tested with TBA assay system. The reduced level of LPO was
associated with the high content of organosulfur compounds in onion oil (Helen et al., 2000). According to Klanns-Dieter, (1983), the \textit{A. cepa} L. dialkyl disulfides and their thiols and oxides trap electrons from other system thus preventing the formation of superoxides and also scavenge other free radicals.

Grudzinski et al., (2001) had reported that DAS obtained from \textit{A. sativum} L. decreases LPO in mice infected with \textit{Trichinella spiralis} which was consistent with previous reports that showed the trapping of methyl and peroxyl radicals by DAS (Fanelli et al., 1998) and modulates glutathione-S-transferase (GST) activity (Munday and Munday, 2001).

5.4.2 Free Radical Scavenging as Second Line Antioxidant Defence

The objective of this defence mechanism is to inhibit the propagation of free radical chain. In specific free radical scavenging activity, antioxidants suppress the formation of free radical chain inhibiting the chain propagation while non-specific free radical scavenging activity targets to scavenge free radicals in the antioxidant system. Both DPPH radical scavenging assay and xanthine/xanthine oxidase (XOD) superoxide scavenging assay are specific free radical scavenging systems while the TEAC assay and FRAP assay are examples of the non-specific free radical scavenging assay (Vimala, 2008). In the present study, the second line of antioxidant defence was evaluated using the DPPH assay and the reducing power assay.
Specific Free Radical Scavenging

21 crude extracts obtained from the selected *Allium* spp. were tested for their specific free radical scavenging ability using DPPH radical. The DPPH assay was chosen to measure the free radical scavenging ability of the crude extracts obtained from the selected *Allium* plants because it is a simple and rapid way of determining specific anti-radical activities of antioxidants. The DPPH assay can accommodate a large number of samples and was sensitive in detecting natural compounds at low concentrations (Russo *et al*., 2005) and it is independent of sample polarity (Koleva *et al*., 2002). However, the DPPH assay was reported to be dependent of the structural conformation of the antioxidant and the scavenging activity was seen to be corresponding with the number of available hydroxyl groups in the antioxidant (Kosar *et al*., 2007).

The pre-screening of the crude *Allium* extracts had identified a few extracts which were considered to be active by demonstrating the ability to decrease in the initial amount of DPPH radicals by 50% or more. At 5 mg/ml, 14.29% from the total *Allium* crude extracts managed to show potential in scavenging the DPPH radicals while others did not show promising antioxidant activity. 14.29% of petroleum ether extracts and 28.57% of chloroform extracts exhibited 50% or more DPPH inhibition while no methanol *Allium* extracts were effective in inhibiting the DPPH radical.

In the presents study, the petroleum ether extracts of *A. tuberosum* Rottl. (daun kuchai) and chloroform extracts of *A. cepa* L. and *A. ursinum* L. had showed potential free radical scavenging ability. Petroleum ether extracts of *A. tuberosum* L. (daun kuchai) and chloroform extracts of both *A. cepa* L. and *A. ursinum* L. had demonstrated IC$_{50}$
values of 3.89 mg/ml, 4.20 mg/ml and 4.55 mg/ml respectively. However, no crude methanol extracts were found to inhibit the DPPH radical. Turkmen et al., (2005) reported that 80% aqueous methanol leek extracts exhibited very low DPPH inhibition activity of 12.2%±1.39. In contrast, Tepe et al., (2005) revealed that the polar sub-fractions of *A. atroviolaceum* was most active in inhibiting DPPH radicals and exhibited IC₅₀ of 79.0 ± 2.75 μg/ml.

Other than that, it was observed that the anti-DPPH radical activities differ in between the leaf and bulbous species in the present study. A hypothesis that there may be chemical compounds in the leafy species that contributed to the scavenging activity of the DPPH radical was suggested. For example, *A. giganteum* L., a genotype of *Allium* is a leaf species. It was reported to have high content of antioxidant enzymes such as SOD and CAT that made it performed great potential in reducing the O₂⁻ and OH° radicals (Stajner et al., 2006).

SOD and CAT play important roles in inducing the decomposition of superoxide to hydrogen peroxide and then to water and oxygen (Rice-Evans et al., 1997a). Therefore, the anti-DPPH radical activities demonstrated by the leaf species in this study may be related to the presence of these antioxidant enzymes which increases the antioxidant potential of the leaf samples compare to its counterparts.

In addition to having typical chemical compounds that give antioxidant properties to the plants, it may also be due to the presence of some pigments in the leaves (i.e. chlorophyll, carotenoid and flavonoid) that contributed to greater anti-free radical
activity of these leaf species. Chloroform extracts of *A. cepa* L. showed the greatest anti-DPPH radical activity with an inhibition rate of 86.27% when pre-screened at 5 mg/ml. *A. cepa* L. contained high content of flavonoids, especially in quercetin which is a powerful radical scavenger. The presence of anthocyanidin as the purple-red pigment on the onion skin may also be the enhancing factor of the antioxidant potential of *A. cepa* L. in scavenging free radicals (Rice-Evans *et al.*, 1997b).

Prior *et al.*, (2005) had reported that antioxidants can inactivate free radicals through two mechanisms which are hydrogen atom transfer and single electron transfer. The hydrogen atom transfer-based systems are rapid and evaluate the free radical quenching activities by antioxidants through hydrogen donation while single electron transfer-based systems are slow and investigate their antioxidative potential to quench free radicals by transferring a single electron.

Although DPPH free radical scavenging assay is a single electron transfer-based system, DPPH can also be neutralized by either direct reduction through electron transfer or radical quenching by hydrogen atom transfer (Prior *et al.*, 2005). The phenolic compounds in *Allium* extracts possess acid protons in their structure and hence, their ability to quench DPPH radicals is very promising in the present study. This was consistent with the results of the present study where the chloroform *A. cepa* L. extract which possess the highest total phenolic content of 820±0.00 mg GAEs/100 g sample exhibited outstanding DPPH radical inhibition at 5 mg/ml with an IC$_{50}$ of 4.2 mg/ml.
In spite of the advantages the plant samples may have, the limitations of the DPPH free radical scavenging assay may also contribute to the inefficiency in measuring the antioxidant activity of the crude *Allium* extracts. The assay is restricted by its non-physiologically low pH value and also a short time of reaction thus not all antioxidants can be measured. The transfer of the H-atom may be slow and the relative percent of decrease in product were most probably measured than the total antioxidant capacity (Jastrzebski et al., 2007). The short changes of this DPPH free radical assay suggested that a single simple assay is not enough to conclude the antioxidant ability of a plant.

Non-Specific Free Radical Scavenging

The petroleum ether, chloroform and methanol extracts of the selected *Allium* spp. were subjected to the reducing power assay in attempt to evaluate their non-specific free radical scavenging activity. In this assay, the presence of reductants (i.e. antioxidants) would reduce the Fe$^{3+}$/ferricyanide complex to the ferrous form, Fe$^{2+}$ resulting in the formation of the Perl’s Prussian blue from reaction mixture of various shades of green and blue. The reducing power of each crude extract was reflected by the absorbance which was monitored by a spectrophotometer at 700 nm and the crude extracts’ potential as an antioxidant could be determined (Chung et al., 2002). In the present study, ascorbic acid was used as standard positive reference.

Overall in the present study, the methanol *Allium* extracts exhibited better reducing powers when compared to its petroleum ether and chloroform extracts. At the highest concentration of 20 mg/ml, the reducing powers of methanol extracts ranged from 0.588A to 2.492A while chloroform extracts with reducing powers of between 0.229A
and 1.678A followed by petroleum ether extracts with the poorest reducing powers of 0.063A to 0.529A. It was observed that in this non-specific free radical scavenging system, methanol *Allium* extracts exhibited the most promising antioxidant activities followed by chloroform extracts and then petroleum ether extracts.

It was hypothesized that the methanol *Allium* extracts may be electron donors and contained a higher amount of reductants. Electrons or hydrogen atoms may react with free radicals, neutralizing them or transform the free radicals into more stable and non-reactive products subsequently breaking the free radical chain reaction. Again, the presence of phenolic compounds was suspected to be associated with the reducing properties of these crude extracts. For instance, methanol *A. porrum* L. extract exhibited the highest reducing power of 2.492A±0.03 and high total phenolic content of 365±0.00 mg GAEs/100 g sample.

Zhao *et al.*, (2005) showed that phenols are synergistic in their actions and work towards scavenging free radicals, chelating metal ions and repairing DNA damage. In a more recent study, the outer layers of *A. cepa* L. were said to contain high amounts of phenols and this characteristic has enhanced the free radical scavenging activities of the plant (Prakash *et al.*, 2007).

### 5.4.3 Inhibition of Oxidative Destruction as Third Line Antioxidant Defence

According to Vimala (2008), the third line of antioxidant defence involves the termination or sacrifice of antioxidants to arrest oxidative degenerations of large molecules (e.g. enzymes and vitamins) in the human body. The β-carotene bleaching
inhibition assay and tyrosinase inhibitory assay are examples of assays used to evaluate the potential of antioxidants in inhibiting the oxidative damage and also in repairing the antioxidants themselves.

In the present β-carotene bleaching method inhibition assay, the crude Allium extracts were tested for their antioxidative properties as LPO inhibitors. In the absence of an antioxidant, β-carotene undergoes rapid discoloration and the higher the rate of lipid peroxidation, the lighter the orange colour would be of the β-carotene compound. During oxidation, the hydrogen atom located on the carbon-11, between the double bonds, of the active bis–allylic methylene group of linoleic-acid is abstracted subsequently forming the pentadienyl free radicals (Frankel, 1998).

The pentadienyl radicals then attack the highly unsaturated β-carotene molecules in effort to reacquire the lost hydrogen atom. Consequently, the β-carotene molecules lose their conjugation and thus lose their characteristic orange colour and this process was monitored spectrophotometrically at 470 nm (Amarowicz et al., 2004). According to Jayaprakasha et al., (2001), antioxidants neutralize the linoleate free radicals and other free radicals in the system thus preventing the bleaching of β-carotene.

The results of the present study indicated that petroleum ether Allium extracts was better LPO inhibitors than chloroform extracts. The methanol extracts of Allium in the present study were least potential LPO inhibitors. The LPO inhibition potential was regarded as antioxidant activities of the Allium crude extracts. Petroleum ether extracts were the most effective followed by chloroform extracts and then methanol extracts. At 20
mg/ml, the petroleum ether *Allium* extracts exhibited LPO inhibition activities of between 25.20- 91.10% while chloroform extracts showed inhibition of 34.18-90.77%. The methanol *Allium* extracts demonstrated LPO inhibition activities of 26.48-77.37%.

Prior *et al.*, (2005) reported that the liposolubility of the extracts played an important role in determining the diffusion process of the phytochemicals into the lipophilic region of β-carotene-linoleic acid/water emulsion. Lipid-soluble compounds were extracted in petroleum ether of low polarity while water-soluble compounds were extracted into methanol with high polarity. Chloroform with intermediate polarity was reported to extract amphiphilic flavonol aglycones (Kathirvl *et al.*, 2009) and thus, in the present study, chloroform may extract the amphiphilic compounds which have both polar and non-polar regions in their structure (Houghton and Raman, 1998; Lide, 1993).

Another researcher, Joyeux *et al.*, (1995) concluded that the presence of phenolic compounds were associated with LPO inhibition potentials of a plant extract and reported that flavonoid was effective in inhibiting LPO. It was also claimed that the potential of the plant extract was directly attributed to the number of free phenolic hydroxyl groups that are present in the phenolic compound and the ortho-hydroxylation of the B-ring was essential in determining the LPO inhibition effects of the extracts.

According to Nuutila *et al.*, (2003), quercetin, a phenolic compound, was one of the effective LPO inhibitor alongside myricetin and kaempferol in *A. cepa* L. extracts. *A. cepa* L. However, no positive correlation was observed in between the LPO inhibition potentials of the *Allium* extracts and their total phenolic contents in the present study.
For example, although the crude chloroform *A. cepa* L. extract was observed with the highest total phenolic content (i.e. 820±0.00 mg GAEs/100 g sample) as compared to other *Allium* crude extracts, it has also weakly inhibited LPO with 26.68%±4.81 at the highest concentration of extract tested (i.e. 20 mg/ml).

### 5.4.4 Total Phenolic Content of Selected *Allium* spp.

The total phenolic contents of the selected *Allium* spp. were determined using the Folin-Ciocalteau reagent. Gallic acid were used as standard commercial positive references in this chemical composition determination assay and total phenolic content of each of the test subject was expressed as mg GAEs/100 g sample.

Overall, it was observed that the petroleum ether extracts of the selected *Allium* spp. had the least phenolic content while the methanol extracts exhibited the highest content of phenolic compounds. This was consistent with findings that reported that phenolics were more easily dissolve in methanol because the solvent has more polarity (Houghton and Raman, 1998). However, the chloroform extracts of *A. cepa* L. exerted the highest total phenolic content among all other extracts with 820±0.00 mg GAEs/100 g sample.

In the present study, whole dried *A. cepa* L., which includes its skin, was extracted for evaluation purposes and results showed that *A. cepa* L. exhibited the highest total phenolic content (i.e. 820±0.00 mg GAEs/100 g sample). The purple pigment in the *A. cepa* L. skin that is believed to be anthocyanidin (Fossen and Andersen, 2003; Fossen et al., 2003) and our finding is consistent with previous studies which suggested that the pigment colour is an overall expression of phenolics (Cieslik et al., 2006; Sass-Kiss et
however, the phenolic contents of a fruit or vegetable may not be attributed only to its colours because their total content may differ according to its plant genotype (Scalzo et al., 2005).

Many studies have reported that A. cepa L. possess high total phenolic content. For instance, Velioglu et al., (1998), who reported that red onions have higher total phenolic content than other plant materials. The richness of phenolic compounds in A. cepa L. made it stood out with the highest total phenolic content in the present study (Prakash et al., 2007; Fossen and Andersen, 2003). Ismail et al., (2004) have reported that fresh samples of A. cepa L. (shallots) exhibited total phenolic content of shallots 2528±43 mg ferulic acid equivalents (FAEs)/100 g extract while thermally treated samples of shallots showed total phenolic content of 2187±49 mg FAEs/100 g extract. High antioxidant activity of shallots was also reported (i.e. 69.10%±1.10) and may be contributed by its flavonoid contents. In Lin and Tang, (2007), the red onion variety exhibited highest total phenolic content of 310.8±4.9 mg GAEs/100 g fresh matter (FM) among other researched vegetables.

Generally, the more hydroxyl group a phenolic compound has, the greater its antioxidant potential. This is because ortho-substitution of the hydroxyl group alongside the presence of electron donating groups tends to increase the antioxidant potential of phenolic compounds (Rice-Evans et al., 1995). However, the phenolic compounds in each type of vegetable may vary differently in their effectiveness as antioxidant (Robards et al., 1999) due to the fact that total phenolic content may differ in different plant genotype (Scalzo et al., 2005).
Nevertheless, Escarpa and Gonzalez, (2001) had reported that this chemical composition determination assay was not specific towards polyphenols but also to any other substance that could be reduced by the Folin-Ciocalteau reagent creating the possibility of false positive results due to the presence of other compounds. In addition to that, phenolic compounds with different number of phenolic groups respond differently to the reagent (Singleton et al., 1999) thus raising the important role of a pure extract in the assay.

5.4.5 Total Flavonoid Content of Selected *Allium* spp.

The total flavonoid contents of the *Allium* extracts were investigated using AlCl$_3$ solution. Quercetin was used as a standard positive reference and the total flavonoid content of the test subjects were expressed as mg QEs/100 g sample.

Overall results indicated that all *Allium* extracts exhibited low total flavonoid contents. The highest total flavonoid content was exhibited by methanol extract of *A. porrum* L. with 56.48±0.00 mg QEs/100 g sample while the least flavonoid content was exhibited by methanol *A. tuberosum* L. (daun kuchai) extract with 3.60±0.00 mg QEs/100 g sample. The antioxidant properties present can be determined by the presence of flavonoids with a certain molecular structure, especially those with a certain hydroxyl position and it is very much depended on its ability to donate hydrogen atom or electrons to a free radical (Meda et al., 2005).

In the present study, total flavonoid content of *A. cepa* L. was 13.88±0.00 mg QEs/100 g sample for petroleum ether extract, 23.60±0.01 mg QEs/100 g sample for chloroform
extract and for methanol extract of *A. cepa* L., its total flavonoid content was 19.85±0.01 mg QEs/100 g sample. In Lin and Tang, (2007), it was reported that the total flavonoid content of two red variety of *A. cepa* L. were 36.5±7.6 QEs/100 g FM and 56.4 ± 10.3 mg QEs/100 g FM, respectively. The variety of total flavonoid content among *A. cepa* L. in this study and in Lin and Tang, (2007) may be attributed to its plant genotype difference (Scalzo *et al.*, 2005).

The difference in *A. cepa* L. extracts preparation may also affect the outcome of the results. In the present study, whole *Allium* vegetables were cut, dried and grounded before subjecting for infusion with solvents. As for Lin and Tang, (2007), fresh matter of *A. cepa* L. was washed, chopped and squeezed for its juice. The juice was then centrifuged, filtered and lyophilized before usage. However, this AlCl$_3$ method is specific only for flavone and flavonol and the total flavonoid content must be the sum of flavonoid content using the AlCl$_3$ method (Chang *et al.*, 2002).

**5.4.6 Antioxidant Activities of Selected *Allium* spp.**

In the present study, the antioxidant activities and potentials of the *Allium* spp. were observed to vary and it may depend greatly on the chemical compounds that exist in that plants. The different of amount of various volatile and non-volatile compounds in the selected *Allium* spp. studied may be one of the factors that determined the antioxidant activity of the crude extracts. The presence of main organosulfur compounds and their precursors in the crude petroleum ether and chloroform extracts was perhaps associated with their potential as antioxidants.
Allicin, DADS and DATS were identified as main antioxidative compounds (Kim et al., 1997). In previous researches, allicin was reported to have in vivo antioxidative properties in low concentrations and at high concentrations; allicin is a pro-oxidant (Lawson, 1998) but its precursor alliin did not show any antioxidative activity in the linoleic acid peroxidation system (Hirata and Matsushita, 1996).

Generally, *Allium* plants are rich in phenolic compounds and flavonoids can be found in abundance in *Alliums*. These flavonoids can either be present as sugar conjugates or as aglycones. Flavonoids were reported to suppress lipid peroxidation by donating its hydrogen atom to other antioxidants and throughout this process; flavonoids retain their own free radical scavenging properties (McAnlis et al., 1999). Quercetin is a major flavonoid which can be found in abundance in *A. cepa* L. and it exist as conjugated form of quercetin-4’-O-β-glycopyranoside, quercetin-3,4’-O-β-diglycopyranoside and quercetin-3,7,4’-O-β-triglycopyranoside (Sellappan and Akoh, 2002).

Again, the type of solvent used for plant extraction also determines the potential of each extract as antioxidants in different antioxidant systems. In the present study, petroleum ether, chloroform and methanol were used to extract the *Allium* plants and generally, petroleum ether dissolves lipid-soluble (lipophilic) compounds while methanol dissolves mostly water-soluble (hydrophilic) compounds. Chloroform is a solvent with intermediate polarity and as such, a mixture of lipid- and water-soluble compounds may be extracted by it (Kathirvel et al., 2009; Houghton and Raman, 1998; Lide, 1993).
Hydrophilic compounds were said to be less potent in oil-in-water emulsions system than lipophilic compounds while lipophilic compounds less in favour of bulk oils system than hydrophilic compounds. This was explained as polar antioxidants were presumed to be able to accumulate at the air-oil interface or form reverse micelles within the oil, a site where lipid oxidation takes place because of the high concentrations of oxygen and pro-oxidants (Porter, 1993).

Polar antioxidants also tend to partition into the aqueous phase and would not be able to protect the lipid layer in emulsion. Non-polar antioxidants favoured emulsions as these compounds may retain and/or accumulate in the oil-water interface, a place where hydroperoxides at the surface of the droplet interacts with pro-oxidants (e.g. transition metals) in the aqueous phase (Porter, 1993). Hence, this explains why petroleum ether extracts were most effective in oil-in-water emulsions systems such as FTC assay and β-carotene bleaching inhibition assay in consistent with the results of the present study.

However, it is important to know that certain antioxidant may show antioxidant potential but still fail to protect and even sometimes cause damage to the cells. Under certain conditions, these antioxidants may scavenge free radicals but also capable of accelerating damage to other macromolecules such as DNA, proteins and amino acids. This is mainly seen within inhibitors of lipid peroxidation such as BHA. BHA is a powerful lipid peroxidation inhibitor but higher dose of BHA may induce oxidative damage and cancer of the rat stomach (Schildermann et al., 1995).
5.5 Evaluation of Anti-HPV Type 16 E6 Oncoprotein Activities Using ICC Technique

Cancer of the cervix is caused by HPV. In 1995, all relevant data on the carcinogenicity of HPV were evaluated by International Agency for Research on Cancer (IARC) and a conclusion was made upon sufficient evidence that HPV types 16 and 18 are carcinogenic towards human (International Agency for Research on Cancer, 1995).

Based on L1 primers, HPV DNA was detected in more than 90% of specimen using PCR assay suggesting a necessary relation in between HPV and cervical cancer. Among the most frequently found HPV, 53% was type 16, 15% was type 18, 9% was type 45, 6% was type 31 and 3% was type 33. Epidemiologically, HPV type 16 was the most prevalent followed by HPV type 18 (Munoz, 2000). In 2003, it was reported that 15 types of HPV have been identified as high-risk for the development of cervical cancer, three types as probable high-risk, 12 as low risk while three are classified as undetermined risk (Munoz et al., 2003).

HPV type 16 infection is very common and it is the most prevalent (Woodman et al., 2007). The transforming properties of high-risk HPV (i.e. HPV type 16) are governed by the E6 and E7 genes. E6 genes have exerted functions in tumourigenesis, regulation of transcription, telomerase and apoptosis. The E6 gene is situated in the 5’ end of the early region and codes for multifunctional proteins that are involved in the cell growth (Munoz et al., 2006).

In the present study, the anti-HPV type 16 E6 activities of selected Allium spp. were investigated using ICC technique. A three-step indirect method using the LSAB
Peroxidase Kit (Dako) and the DAB Substrate System (Dako) was used to detect and evaluate the E6 expression in the CaSki cells. It is a simple and sensitive staining technique which produces reliable and consistent results. Several critical factors which can greatly affect the outcome of the stain are discussed as follows.

Fixative

One of the first and foremost crucial considerations is fixative. Before localization of antigens within tissues and cells can be done, it is important to know the chemistry of fixatives in order to understand their action and to avoid artifacts. An ideal fixative is a fixative that preserves cellular structure and prevents the loss and/or migration of antigens (Bowers and Maser, 1988). It should also be non-toxic to the cells, economy and cost-effective (Nadji, 2006; Vincek et al., 2003).

The fixative should insolubilize and inactivates the viral antigens in the tissues or cells while at the same time should not denature and/or change immunological reactivity of permeability towards the antibody (Larsson, 1988). Forghani and Hagens (1995) documented that it was important to monitor and optimize the usage of fixative as over-fixation can result in a false negative staining.

Acetone is the most common fixative used for fixing films, smears of cells and unfixed cryostat sections (Kiernan, 1990) and for acetone, the temperature of fixation and concentration of fixative are important factors. Acetone usage for fixation is recommended with varying time and temperatures (e.g. 5-15 min at room temperature, 12-20 min at 4°C, 3-30 min at -60°C or -20°C overnight) (Forghani and Hagens, 1995;
To and Bernard, 1992). Although acetone was used to fix the slides containing the CaSki cells in this study, Cinar et al., (2006) reported that acetone was a poor fixative in preserving general cellular organization. It was suggested that ethanol- and propylene glycol-based fixatives were more suitable for cytology and compatible for general antibody staining (Key, 2006).

**Buffer**

The common buffers recommended for dilution of primary antibodies have physiological pH near 7-7.2. In the present study, PBS pH 7.6 was used as antibody diluent and also for washing purposes. PBS has long been used as a wash buffer and it is inexpensive to produce. The pH of the PBS buffer was higher than recommended and had caused slight background staining.

It was reported by Pace, (2006) that PBS increase non-specific cell staining and should not be used as an antibody diluent unless it was recommended by the manufacturer. This wash buffer may also produce primary antibody shielding and this decreases the specific binding efficiency of the antibody. As such, antibody diluents with Tris-HCl and background reducing agents were recommended.

**Dehydration and Rehydration**

In the present study, after the application of cells onto the microscopic slides, the slides were air-dried and fixed with -20°C acetone. By air-drying the cells, the cells were more firmly attached to the slide (Farmilo and Stead, 2001). It was also recommended by Key, (2006) to dehydrate the slides before storage as it was important to maintain the
cells’ morphological details and/or prevent antigenic changes and also to retain their protein molecules in their natural conformation prior to staining. Slides were then kept in a box at -20°C prior to use.

Before proceeding with the staining protocol, slides were removed and left at room temperature for 30 minutes. After that, slides were immersed in 100% alcohol and then diminishing concentrations of alcohol (i.e. 95%, 90% and 80% alcohol) for two minutes each before equilibrating in PBS for five minutes. The purpose of alcohol rehydration before staining was necessary to restore the permeability of the cells thus improving their immunoreactivity towards antibody and the staining dye (Key, 2001).

Alcohol is coagulant fluid and does not form additive compounds thus permitting good antibody penetration (Farmilo and Stead, 2001). On the other hand, PBS pH 7.6 was used as a ‘retrieval solution’ in this study to retrieve the target antigen (i.e. E6 oncoprotein). However, ‘retrieval solutions’ of pH near 2, 6, 8 or 10 are preferred and a small amount of detergent may help increase the permeability of the cells (Key, 2001).

Endogenous Peroxidase Activity

The presence of naturally occurring endogenous peroxidase enzyme activity is frequently encountered in test samples and if not inhibited or destroyed, it will lead to false positive stains. The pre-treatment of cells with H₂O₂ prior to incubation with primary antibody was important to remove endogenous enzymes that may destroy some viral antigens (Van Noorden, 1986). The most common procedure for removing the endogenous peroxidase activity was by incubating the cells in 3% H₂O₂ for 5-10
minutes (Wendelboe and Bisgaard, 2006) and in the present study, CaSki cells were treated with 3% \( \text{H}_2\text{O}_2 \) for 10 minutes.

Other than 3% \( \text{H}_2\text{O}_2 \), methanolic \( \text{H}_2\text{O}_2 \) and sodium azide \( \text{H}_2\text{O}_2 \) mixtures can also be used to remove endogenous peroxidase activities. However, methanolic \( \text{H}_2\text{O}_2 \) was not recommended if cell surface markers need to be stained (Wendelboe and Bisgaard, 2006).

**Antibody**

In the present study, the anti-HPV type 16 E6 oncoprotein monoclonal antibody was used to detect the E6 oncoprotein expression which was indicated by the reddish-brown stain observed in the CaSki cells. This antibody is selective, meaning the antibody only binds to the antigens against which they were raised (Boenisch, 2006a); in this case the target antigen was E6 oncoprotein. The anti-HPV type 16 E6 oncoprotein monoclonal antibody detects the HPV-infected cells as it has high affinity for the E6 oncoprotein expressed by the cells (Houghton and Scheinberg, 1991).

It was also necessary to ensure that the antibodies should be stored promptly upon receipt according to the storage recommendations by the manufacturer. It was advised to store the antibodies at the temperature of 2-8°C prior to use because freezing and thawing would affect the performance of the antibodies (Boenisch, 2006a). The anti-HPV 16 E6 monoclonal antibody in this study was stored at 4°C upon receipt and prior to use.
Antibody titer and dilution factor were also important in determining the quality of the staining. An optimum antibody titer should result in a maximum specific staining with the least amount of background under specific test conditions with the highest dilution of a monoclonal antibody (Boenisch, 2006c). According to Forghani and Hagens, (1995), an ideal antibody should have a high titer, high affinity and avidity as it should only react with the viral antigen and not with the host cell or growth medium components. Antibody titers may vary accordingly from 1:100 to 1:2000 for polyclonal antibody and 1:10 to 1:1000 for monoclonal antibodies in cell culture supernatants. For monoclonal antibodies in ascites fluid, the antibody titer may reach up to 1:1,000,000 (Boenisch, 2006c). The E6 expression was detected using the anti-HPV 16 E6 monoclonal antibody with a 1:50 dilution in the present study.

Incubation

A consistent intensity of stain is essential to detect and evaluate the expression of the target antigen. In this case, the staining intensity was used to indicate the amount of the target antigen (e.g. E6 oncoprotein) present in the CaSki cells. In the present study, initially the slides were incubated at room temperature (27°C) but however, the stain intensity appeared to be inconsistent and CaSki cells were tested negative for E6 oncoprotein. This did not correlate with previous findings citing the abundance of E6 in HPV infected tissues and derived cell lines. Experiments were then repeated whereby the incubation temperature was increased to 37°C. At this temperature, equilibrium of antigen-antibody reactions is reached more quickly as compared to room temperature (Boenisch, 2006c).
It is also crucial to ensure that saturation of antigen with primary antibody is reached during incubation. Generally, the incubation times recommended is within 24 hours, with 10-30 minutes being the most commonly used incubation time. To evaluate the E6 suppression by the Allium crude extracts, the CaSki cells were incubated with the primary antibody (i.e. anti-HPV type 16 E6 oncoprotein monoclonal antibody) for 90 minutes. Shorter incubation period is recommended for high affinity and/or high titer antibodies to reach equilibrium while longer incubation period is recommended for low affinity and/or low titer antibodies before the antigen is saturated with the antibody (Boenisch, 2006c).

During incubation, it is also important to incubate slides in a humid incubation chamber at all times to prevent evaporation and drying of cells. This was because drying out caused non-specific antibody binding and therefore high background staining (Boenisch, 2006c). In the present experiment, the slides were incubated in a shallow plastic box with sealed lid and lined with wet tissue paper at the bottom.

**Mounting Medium**

As some of the precipitated chromogens are soluble in organic solvents, a suitable mounting medium is essential to not only preserve the immunoenzymatically stained specimen but also adhere well to the slide and prevents fading of the chromogen for easy visualization. Polyvinyl alcohol (PVA)/glycerol formulation is a good general-purpose mounting medium (Ono et al., 2001) that is capable of retaining the colour intensity of slides stained with the peroxidase chromogen DAB for more than ten years
Glycergel was used as a mounting medium in the present study.

5.5.1 Anti-HPV Type 16 E6 Oncoprotein Activities of Selected *Allium* spp.

Normal human cell cultures do not have a long life span. These cells stop growing in cultures after some time. However, the immortalization of these cells via expression of some oncogenes allowed these cells to proliferate. Indefinitely, this is an important step in the progression to full malignancy. Oncogenes are critical in the carcinogenesis of cancers. Hence, the function of these oncogenes should be suppressed to prevent the progression of carcinogenesis. Many studies have successfully implemented the use of natural products to suppress the expression of oncogenes as a step to prevent the progression of carcinogenesis.

For example, acetone extracts of *Angelica sinensis* exhibited suppression activity against the Bcl-2 oncoprotein. The suppression of Bcl-2 activates apoptosis and inhibited the proliferation of A549 cells (Cheng *et al*., 2004). Another compound, curcumin had significantly suppressed the DMBA-TPA induced *ras* and *fos* oncogenes expression in mouse skin (Limtrakul *et al*., 2001). It was also reported that curcumin from *Curcuma longa* L. up-regulates the Bax proto-oncogene which then induce the release of cytochrome c from the mitochondria. Subsequently, caspase-3 was activated inducing apoptosis in Ehrlich’s ascites carcinoma (EAC) cells (Pal *et al*., 2001). In a more recent study, jaceosidin, a compound isolated from *Artemisia argyi* had exhibited inhibitory effects against the binding between HPV 16 E6 oncoprotein and p53 gene and between HPV 16 E7 oncoprotein and pRb gene (Lee *et al*., 2005).
The carcinogenic potential of E6 from high-risk HPV in multiple tissues which these oncogenes were expressed included the induction of squamous cell carcinomas. These forms of cancer were closely associated with anogenital papillomaviruses (Coussens et al., 1996). The E6 oncogene is also vital in the carcinogenesis of the cervix and the expression HPV 16 E6 oncoprotein can immortalize human epithelial cells (Shay et al., 1993). Therefore, it was crucial to inhibit the function of E6 oncogene to prevent HPV infections.

Previous research had demonstrated the use of organosulfur compounds from A. sativum L. (garlic) to suppress the expression of oncoproteins. According to Arora et al., (2006), DAS found in A. sativum L. was reported to downregulate the expression of the p21/ras oncoprotein in DMBA-induced mouse skin tumours. Earlier studies showed that another organosulfur compound, DADS was claimed to inhibit the p21H-ras oncogene processing in vivo causing H-ras oncogene-transformed tumour growth arrest (Singh, 2001; Singh et al., 1996).

The p53 tumour suppressor gene plays an important role in inducing apoptosis. Early cell death does not allow the virally infected cells to replicate and produce progeny virus. Generally, p53 is activated when the cell is infected with by virus and several viruses were found to specifically inhibit the p53-dependent apoptosis such as adenoviruses. As it was reported that the E6 oncoprotein may bind to E6-associated protein (E6AP) to cause the degradation of p53 via the ubiquitination pathway (Howley, 1996), it was proposed that the E6 oncoprotein would suppress apoptosis (Rapp and Chen, 1998).
The HPV type 16 E6 oncoproteins in cancer of the cervix cell line, C-33A was reported to transactivate the promoters of SV40 and HIV in a p53-dependent pathway (Kang et al., 2003). In a more recent study, jaceosidin, a compound isolated from Artemisia argyi was reported to inhibit the binding between E6 and p53 and also the binding between E6AP and p53 and induces apoptosis (Lee et al., 2005).

In the present study, the anti-HPV type 16 E6 monoclonal antibodies were used to detect the presence of E6 oncoprotein in the human cervical cancer-derived HPV type 16-containing cell line, CaSki obtained from American Tissue Culture Collection (ATCC). The negative controls for this study were Caski cells that were not incubated with anti-HPV type 16 E6 antibody and the CaSki cells that were not treated with plant extracts. (Plate 5.1(a-b)). Untreated CaSki cells that were not incubated with the anti-HPV monoclonal antibody did not demonstrate any reddish-brown stain because the E6 oncoprotein was not targeted (Plate 5.1(a)).

On the other hand, untreated CaSki cells expressed reddish-brown stain in the cell cytoplasm and/or nuclear regions. The presence of the stain indicates the presence of E6 oncoprotein. The darker the staining intensity, the higher the amount of E6 expressed (Plate 5.1(b)). The intensity of reddish-brown stains detected in the CaSki cells are classified as no stain (-), mild (+), moderate (++), strong (+++) and very strong (++++).
Plate 5.1(a) Untreated CaSki cells without anti-HPV type 16 E6 monoclonal antibody incubation (40X); and (b) Untreated CaSki cells with anti-HPV type 16 E6 monoclonal antibody incubation (40X)

CaSki cells were treated with crude extracts of the selected *Allium* plants at various concentrations of 200 $\mu$g/ml, 150 $\mu$g/ml, 100 $\mu$g/ml, 50 $\mu$g/ml, 25 $\mu$g/ml, 12.5 $\mu$g/ml, and 1 $\mu$g/ml. The cells were incubated for 72 hours at 37°C before subjected to ICC. The staining results were compared to the negative controls and among various concentrations of the same crude extract. The E6 oncoprotein expression was denoted by reddish-brown stains found mostly in the cytoplasm region of CaSki cells, correlating with the findings of a previous study (Nurhayati *et al*., 2003).

Nevertheless, the reddish-brown stain that was expressed showed that there was E6 oncoprotein expression by the CaSki cells where the more intense the reddish-brown stain, the higher the expression of E6 oncoprotein. Suppression of E6 oncoprotein by plant extracts should therefore result in a decrease in the intensity of the reddish-brown stain.
Crude Petroleum Ether Extracts

Generally, all petroleum ether extracts exhibited moderate to strong inhibition activity against HPV 16 E6 oncoprotein in CaSki cells. The expression of E6 in CaSki decreased with the increasing concentrations of *Allium* extracts. In the present study, 57.14% of the samples tested demonstrated anti-E6 activities indicated by staining intensities of moderate (++) and below, at the lowest concentration of 1 µg/ml. Among these extracts, three of the petroleum ether extracts obtained from *A. fistulosum* L., *A. porrum* L. and *A. tuberosum* Rottl. (daun kuchai) were found to effectively suppress the E6 oncoprotein in CaSki cells. At concentration 200 µg/ml, petroleum extracts of *A. cepa* L. and *A. ursinum* L. were the poorest in inhibiting the expression of E6 oncoprotein.

Based on the suppression trend and intensity of reddish-brown stains, overall it was observed that the petroleum ether extracts of *A. fistulosum* L. and *A. tuberosum* Rottl. (daun kuchai) were the most active in suppressing the expression of E6 oncoprotein. The suppression of E6 oncoprotein by the other *Alliums* was also observed in a decreasing manner: *A. porrum* L. > *A. tuberosum* Rottl. (bunga kuchai) > *A. cepa* L. > *A. sativum* L. > *A. ursinum* L.. Overall, CaSki cells were still intact after treatment with petroleum ether extracts of *Allium* plants. However, mild lysis was seen in cells treated with 25 µg/ml of petroleum ether *A. sativum* L. and both variants of *A. tuberosum* Rottl. extracts.
Results also revealed that petroleum ether extracts of *A. fistulosum* L., *A. porrum* L. and *A. tuberosum* Rottl. (daun kuchai) were more HPV 16 E6 suppressive when compared to their chloroform and methanol extracts.

**Crude Chloroform Extracts**

Based on the suppression trend and intensity of reddish-brown stains, it was generally observed that the chloroform extracts of *Allium* evaluated showed moderate anti-HPV 16 E6 oncoprotein activities in a dose-dependent manner where the higher the concentration of extracts used, the greater the inhibition effects on the E6 oncoprotein expression.

The results of the present study revealed that at the concentration of 1 µg/ml, the four chloroform extracts which exhibited anti-E6 activities indicated by staining intensities of moderate (++) and below were *A. fistulosum* L., *A. sativum* L. and both variants of *A. tuberosum* Rottl.. 14.29% of chloroform *Allium* extracts tested was effective in strongly suppressing the expression of HPV 16 E6 oncoprotein in CaSki cells at the lowest concentration of 1 µg/ml.

In the present study, the results showed that chloroform *A. sativum* L. extract was most effective in suppressing the expression of E6 oncoprotein in CaSki cells, followed by *A. fistulosum* L., two variants of *A. tuberosum* Rottl., *A. porrum* L., *A. cepa* L. and *A. ursinum* L. in a decreasing manner. At concentration 200 µg/ml, all chloroform extracts except *A. ursinum* L. effectively inhibited the expression of HPV 16 E6 oncoprotein in CaSki cells.
CaSki cells treated with chloroform extracts of *A. fistulosum* L. and both variants of *A. tuberosum* Rottl. showed lysis at all concentrations of extracts applied. *A. sativum* L. exerted mild lysis effect on the CaSki cells at concentrations 25 μg/ml and below. Overall, the chloroform extract with the most consistent HPV 16 E6 oncoprotein suppression activity was *A. sativum* L. and its anti-E6 oncoprotein expression was better when compared to its petroleum ether and methanol counterparts.

**Crude Methanol Extracts**

All methanol extracts of *Allium* spp. exhibited moderate to strong inhibition of HPV 16 E6 oncoprotein. Based on the immunocytochemistry results, the methanol extracts suppressed the E6 oncoprotein in a dose-dependent manner where E6 suppression increased with the increasing concentrations of *Allium* extracts. At the lowest concentration of 1 μg/ml, 42.86% of the methanol extracts exhibited moderate to strong inhibition of HPV 16 E6 oncoprotein in CaSki cells.

Overall, methanol extract of *A. tuberosum* L. (bunga kuchai) was the most prominent extract in demonstrating significant E6 inhibition in the cells while *A. porrum* L. methanol extract had contrastingly increased the expression of E6 oncoprotein. The anti-E6 oncoprotein activities of other methanol *Allium* extracts are as follows: *A. tuberosum* Rottl. (daun kuchai) > *A. ursinum* L. > *A. fistulosum* L. > *A. sativum* L. > *A. cepa* L. in a decreasing manner.

Unlike the rest, the methanol extracts of *A. porrum* L. demonstrated opposing activity where CaSki cells treated with methanol *A. porrum* L. extracts were induced to express
E6 oncoprotein in a dose-dependent manner from 1 µg/ml to 200 µg/ml. At the lowest concentration of extracts 1 µg/ml, the reddish-brown stain intensity was moderate (+) indicating moderate amounts of E6 oncoprotein. From 100 µg/ml on, the reddish-brown stain intensity was observed to be strong (+++) indicating high amounts of E6 oncoprotein. No literature data was found in relation to this phenomenon.

Generally, the CaSki cells treated at all concentrations of methanol extracts of A. cepa L., A. porrum L., A. tuberosum Rottl. (daun kuchai) were intact. However, methanol A. fistulosum L. extract had caused mild lysis in the cells at all concentrations of extracts. Mild lysis was also seen in CaSki cells treated with concentrations 50 µg/ml and below of methanol A. sativum L., 50 µg/ml and above methanol extracts of A. ursinum L. and 25 µg/ml and above methanol A. tuberosum Rottl. (bunga kuchai) extracts. In the present study, methanol extracts of A. ursinum L. and A. tuberosum Rottl. (bunga kuchai) were better in suppressing E6 activity than its petroleum ether and chloroform counterparts.

While the evidence obtained in this study is compelling, the methanol A. porrum L. extract was the only extract that showed potential in increasing the E6 oncoprotein expression in the CaSki cells. The discrepancy may be explained that there may be presence of an active compound in the methanol extracts of A. porrum L. which activates or induces the E6 oncoprotein production which have yet to be identified. The application of sophisticated screening system such as ELISA and Western Blot may be useful in providing a more stable basis for comprehensive analysis of the cellular
function studies of HPV 16 E6 oncoprotein as well as the development of an anti-HPV drug in the future.

5.5.2 Comparison of Anti-HPV Type 16 E6 Oncoprotein Activities of Selected Allium spp.

The anti-HPV study was focused on the HPV type 16 E6 oncoprotein because HPV 16 is the most prevalent causal factor for cervical cancer and E6 is one of the two oncoproteins causing the cancer. Generally, data suggested that the petroleum ether, chloroform and methanol extracts of the selected Allium spp. demonstrated different suppression effects against HPV 16 E6 oncoprotein. Overall, the petroleum ether Allium extracts were more suppressive than chloroform and methanol extracts against the expression of HPV 16 E6 oncoprotein. Based on the staining results, petroleum ether and methanol extracts exhibited average to strong E6 inhibition activities while chloroform extracts showed moderate inhibition against HPV 16 E6 oncoprotein in CaSki cells.

The difference of polarity among the solvents indeed played an important role in determining what type of bioactive compounds extracted. For instance, petroleum ether is a non-polar solvent and dissolves lipid-soluble products such as volatile oils, waxes, fats, pigments and some important products of Allium spp. such as DADS, DATS and the lacrimatory precursor of organosulfur compounds, S-propenylcysteine sulfoxide (Bianchini and Vainio, 2001; Houghton and Raman, 1998).

Chloroform is more capable of extracting slightly more polar compounds such as alkaloids, aglycones, carotenoids and some volatile oils. Methanol with higher polarity
extracts sugars, amino acids, glycosides and phenolic compounds (Houghton and Raman, 1998). Due to the complex mixture of phytochemicals in crude extracts, the anti-E6 activity exerted could not be detected precisely for a specific compound. Therefore, it is beneficial if these crude extracts can be studied comprehensively and specific compounds can be isolated and identified for further evaluations.

Overall results indicated that all the *Allium* exhibited moderate HPV 16 E6 oncoprotein inhibition activities among their petroleum ether, chloroform and methanol extracts. Nonetheless, there were plants that were very significant in suppressing E6 oncoprotein in the CaSki cells in one solvent extract but performed weakly in other solvent extracts in inhibiting the E6 activity.

For instance, petroleum ether extracts of *A. fistulosum* L., and *A. porrum* L. were outstanding in suppressing the HPV 16 E6 oncoprotein in CaSki cells but chloroform *A. fistulosum* L. only showed moderate E6 inhibition activity. Methanol extracts of *A. porrum* L. was otherwise increasing the expression of E6 oncoprotein in the cells. No single plant was seen with all of its solvent extracts became strong inhibitors of E6 oncoprotein expression.

The present results also indicated that 38.10% of the *Allium* extracts did not cause lysis in the CaSki cells while the rest of the extracts induced lysis in CaSki cells. In the main, 42.86% of both petroleum ether and methanol extracts were not cytotoxic against the cells thus no lysis was observed. Six of the chloroform *Allium* extracts induced lysis in CaSki cells. 57.14% of the methanol extracts had induced mild lysis among the CaSki
cells. Moderate lysis was induced by the chloroform *A. fistulosum* L. extract in the cells. *A. cepa* L. was the only plant that did not affect the integrity of CaSki cells for all three extracts while *A. fistulosum* L., *A. sativum* L. and *A. tuberosum* Rottl. (bunga kuchai) were *Alliums* that caused cell lysis for all three extracts.

### 5.6 Conclusion

In the present study, the cytotoxic, antioxidative and anti-HPV 16 E6 oncoprotein activities were successfully screened in 21 crude extracts obtained from seven selected *Allium* plants. The results showed that the *Alliums* were not cytotoxic against MRC-5, CaSki and HCT-116 cell lines.

Overall in the present study, *Alliums* exhibited potential antioxidative activities. The petroleum ether *Allium* extracts exhibited promising LPO inhibition activities while the methanol extracts were more efficient reducing agents. Effective DDPH radicals scavenging activity was demonstrated by petroleum ether extracts of *A. tuberosum* Rottl. (daun kuchai) and chloroform extracts of both *A. cepa* L., and *A. ursinum* L.. The LPO inhibition activities demonstrated may be related to the presence of lipid-soluble organosulfur compounds while the water-soluble organosulfur compounds may be responsible in contributing to the reducing powers of the *Alliums*. Therefore, it can be concluded that the crude *Allium* extracts contained compounds exhibiting antioxidant activities through various mechanisms.

In the present study, there was no exact correlation between the antioxidative activities of *Allium* spp. and their total phenolic and flavonoid contents. However, chloroform
extracts of *A. cepa* L. showed positive correlation between their DPPH radical scavenging and reducing power activity, and its total phenolic contents. This positive correlation may be due to the additional presence of anthocyanidin, a purple-red flavonoid pigment found in their outer layer skin which other selected *Allium* spp. tested do not have. Thus, further comprehensive investigations ought to be done to warrant the potential of this plant.

In the present study, the petroleum ether, chloroform and methanol extracts of *A. cepa* L., *A. fistulosum* L., *A. sativum* L., *A. ursinum* L. and both variants of *A. tuberosum* Rottl. exhibited positive anti-HPV type 16 E6 activities. As for *A. porrum* L., only its petroleum ether and chloroform extracts were active in suppressing E6 activities. This is a very interesting finding and further investigation should include identifying and isolating the bioactive principles. However, the methanol extract of *A. porrum* L. demonstrated the promotion of E6 expression. This unexpected finding deserves to be further investigated.

It is reasonable to promote *Alliums* in cancer chemoprevention since it exhibit anti-HPV and strong antioxidative activities and at the same time not cytotoxic to human cell lines. Hence, further studies should include testing isolated active compounds from *Allium* for their anti-HPV activities.
REFERENCES


**APPENDIX A**

Determination of Total Phenolic and Flavonoid Content of Selected *Allium* spp.

**A) Total Phenolic Content**

Gallic acid as standard calibration curve

<table>
<thead>
<tr>
<th>Concentration of Gallic Acid (mg/L)</th>
<th>Absorbance Reading 1</th>
<th>Absorbance Reading 2</th>
<th>Absorbance Reading 3</th>
<th><em>Average Reading</em></th>
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</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000 ± 0.00</td>
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<tr>
<td>50.0</td>
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<td>0.476 ± 0.00</td>
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* Absorbance reading as mean of triplicate experiments ± standard deviation (SD)

![Graph showing the calibration curve for gallic acid with equation y = 0.001x and R² = 0.9999]
B) Total Flavonoid Content

Quercetin as standard calibration curve

<table>
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<tr>
<th>Concentration of Quercetin (mg/ml)</th>
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<th>*Average Reading</th>
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<td>0.528</td>
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</tbody>
</table>

* Absorbance reading as mean of triplicate experiments ± standard deviation (SD)

![Graph showing the calibration curve for Quercetin with the equation y = 0.0104x - 0.0004 and R² = 0.9982](image-url)