Chrysin in PI3K/AKT and Other Apoptosis Signalling Pathways, and its Effect on HeLa Cells

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1 Introduction

Over 4,000 flavonoids have been identified as a broad class of plant secondary metabolites (Kuntz et al., 1999; Samarghandian et al., 2011). Flavonoids originate naturally in plants are synthesised from amino acid phenylalanine by the phenylpropanoid metabolic pathways, such as shikimate and arogenate pathways (Harborne & Turner, 1984; Soumajit & Ramesh, 2010). Flavonoids display brilliant colours in the flowering parts of plants (Clifford & Cuppert, 2000). These compounds exhibit protective effects against microorganisms, UV light and spread of diseases; and are dietary polyphenols essential to both human and animal health (Khoo et al., 2010; Manika et al., 2012). Flavonoids cannot be synthesised by humans or animals. In contrast, plants have to manufacture what the plants need, not merely to grow, but to defend, protect and heal from stress that would help humans or animals under similar circumstances. Flavonoids are classified into at least 10 main chemical groups. Those are flavanones, flavones, isoflavonoids, flavanols, anthocyanins and flavonols (Table 1) (Cook & Samman, 1996; Bravo, 1988; Aherne & Obrien, 2002; Lakhanpal & Rai, 2007). Flavonoids are ubiquitously present in the green plants and human diet, including in vegetables, fruits, honey, nuts, seeds, coffee, tea and wine (Ho et al., 1992; Kuntz et al., 1999), but the subclasses of flavonoid do not seem to be uniformly distributed (Neuhouser et al., 2009). In addition, flavonoids content is influenced by surrounding factors, such as season, sunlight, climate, food preparation and processing. The average daily flavonoid intakes seem to vary greatly between countries where the lowest intakes (2.6 mg/d) are in Finland and the highest intakes (68.2 mg/d) are in Japan (Nijveldt et al., 2001). Nonetheless, it is extremely difficult to estimate the daily human intake of flavonoids, especially the lack of standardized analytical methods (Scalbert & Williamson, 2000). Similar to daily intake, it is also quite complex to assess and quantify the bioavailability of flavonoids due to the significantly difference between metabolized flavonoids and native compounds present in blood (Russo et al., 2007). It is important to note that flavonoids are biologically active compounds, which contain multiple potent biological effects, including anti-allergic, anti-thrombotic, anti-inflammatory, anti-oxidant, anti-viral and anti-cancer activities (Samarghandian et al., 2011). The flavonoids modulate also the function of sex hormones and the hormones’ receptors. Certain flavonoids, such as isoflavone genistein, are estrogenic (Wang et al., 1996; Zand et al., 2000), whereas others, such as chrysin, can interfere with steroid synthesis and metabolism. Although flavonoids are often called phytoestrogens, only a limited number are estrogen receptor agonists, indeed. In contrast, many flavonoids are known to interfere to a greater or lesser extent with various P450 enzymes, including those in steroidogenesis. Epidemiological studies have shown that the consumption of flavonoids is associated with a low risk of cancer (Block et al., 1992). The cancer chemopreventive properties of flavonoids have become an important topic of investigation. Flavonoids are safe to use and are associated with low toxicity, making these compounds (constituents) potential chemopreventive agents against several types of human cancer, in general. Moreover, the abilities of the compounds to inhibit the cell cycle, cell proliferation and oxidative stress; to activate detoxification enzymes and apoptosis induction; to influence signal transduction pathway, as well as to enhance the immune system, making the compounds ideal candidates for cancer chemoprevention (Yao et al., 2004; Birt et al., 1999). Therefore, flavonoids have gained importance in the pharmaceutical field. The flavonoids are widely available as nutritional supplements, which could be extensively used as primary sources of complementary and alternative health care products or economic in-house regimens for cancer patients. It is still controversial whether all natural flavonoids are beneficial for the prevention and treatment of human cancers. The biological actions of flavonoids depend on the structure and the
### Table 1: Classification of flavonoids. The flavonoid in bold is under our current study. This table is derived from the article by Lakhanpal & Rai, 2007.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Compounds</th>
<th>Food Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavonols</strong></td>
<td>Quercetin, Kaempferol, Myricetin, Isorhamnetin, Quercetagetin</td>
<td>Yellow onion, Curly kale, Leek, Cherry, Tomato, Broccoli, Apple, Green &amp; Black tea, Black grapes, Blueberry, Olives, Lettuce, Parsley</td>
</tr>
<tr>
<td><strong>Flavones</strong></td>
<td>Tangeretin, Heptamethoxyflavone, Nobiletin, Sinensetin, Quercetogetin. <strong>Chrysin</strong>, Apegenin, Luteolin, Disomentin, Tricetin</td>
<td>Parsley, Celery, Capsicum pepper, Apple skins, Berries,</td>
</tr>
<tr>
<td><strong>Flavanones</strong></td>
<td>Naringenin, Eriodictyol, Hesperetin, Dihydroquercetin, Dihydrofisetin, Dihydrobinetin</td>
<td>Orange juice, Grapefruit juice, Lemon juice</td>
</tr>
<tr>
<td><strong>Flavanols</strong></td>
<td>Silibinin, Silymarin, Taxifolin, Pinobanksin</td>
<td>Cocoa, Cocoa beverages, Chocolates</td>
</tr>
<tr>
<td><strong>Catechins</strong></td>
<td>(+) Catechin, Gallocatechin, (-) Epicatechin, Epigallocatechin, Epicatechin 3-gallate, Epigallocatechin 3-gallate</td>
<td>Chocolate, Beans, Apricot, Cherry, Grapes, Peach, Red wine, Cider, Green &amp; Black tea, Blackberry</td>
</tr>
<tr>
<td><strong>Isoflavones</strong></td>
<td>Daidzein, Genistein, Glycitein</td>
<td>Soy cheese, Soy flour, Soy bean, Tofu</td>
</tr>
<tr>
<td><strong>Anthocyanins</strong></td>
<td>Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin</td>
<td>Blueberry, Blackcurrant, Black grapes, Cherry, Rhubarb, Plum, Strawberry, Red wine, Red cabbage</td>
</tr>
</tbody>
</table>

structural properties may be crucial for the actions of the compounds (Jin et al., 2007 & Yang et al., 2006).

### 2 Chrysin

Chrysin (5,7-dihydroxyflavone) is the natural and biologically active flavones group of flavonoids. It is the focus of this review (Nijveldt et al., 2001; Awad et al., 2003; Zheng et al., 2003; Ernst, 2006; Huang et al., 2006; Scheck et al., 2006; Cole et al., 2008; Kale et al., 2008; Parajuli et al., 2009) (Figure 1). Chrysin can be extracted from plants, such as passion flower (Beaumont et al., 2008), silver linden, honey and propolis of some geranium species (Samarghandian et al., 2011). It has been reported that chrysin can also be found in a species of mushroom, *Pleurotus ostreatus* (Anandhi et al., 2013). In addition to being food flavourant and pigment, chrysin has been identified as food constituent that plays important biological roles in nitrogen fixation and chemical defences (Khoo et al., 2010). The chemical structure of chrysin shares the common flavone structure, which composed of fused A and C rings, and a phenyl B ring attached to position 2 of the C ring, with hydroxyl groups at position 5 and 7 of ring A (Figure 1). Chrysin has been shown to be an analogue of galangin, baicalein, apigenin, kaempferol, luteolin and quercetin. However, its anti-cancer properties have seldom been studied in detail compared with other flavonoids. Preliminary, chrysin is observed to have lowered cytotoxic activity in vitro in certain human cancer cells, compared with other flavonoids. However, the potential apoptotic effect of chrysin has been reported in human cervical cancer cells, leukemic cells, esophageal squamous carcinoma cells, malignant glioma cells, breast carcinoma cells, prostate cancer cells, Non-Small Cell Lung Cancer (NSCLC) cells and...
5,7-dihydroxylflavone

Molecular formula: C_{15}H_{10}O_{4}

Molecular mass: 254.24 g/mol

Figure 1: General information and chemical structure of chrysin.

Chrysin is also reported as a potent inhibitor of aromatase which is responsible for blocking the conversion of androgen to estrogens (Sanderson et al., 2004; Khoo et al., 2010). Estrogens are known as cell proliferators and their metabolites, such as catechols, are carcinogens. A local expression of aromatase is suggested to be closely connected with tumour initiation, promotion and progression (Chen, 1998). Therefore, chrysin seems to be a promising way for treatment towards steroid hormone-dependent cancers (breast and prostate), as well as preventing menopausal symptoms by blocking the aromatase activities (Nga & Walle, 2007). However, its blockade should not interfere with the production of other steroids (Hodek et al., 2002). The situation may be different when high intake of chrysin is made, whereby it may result accumulation of flavonoid in tissue that are sufficiently high to inhibit aromatase activity. The sharp decreases of aromatase activity in females may also lead to disruption of the menstrual cycle (Brodice et al., 1989) and loss of bone density (Turner et al., 1994). As for men, decrease estrogen synthesis may also result in deleterious effects on bone homeostasis (Vanderschueren et al., 1998) and disruption of spermatogenesis (Carreau et al., 2003). Although, aforementioned of flavonoids plays a role in
modulating the function of sex hormones and their receptors. However, the function of chrysin as a putative inhibitor for aromatase is poorly elucidated. Most of the studies reported that chrysin was used to raise or stimulated testosterone concentration (Kellis et al., 1984; von Brandenstein et al., 2008) that increased it marketing values by health food stores and is used by many body builders (Nga & Walle, 2007). The oral bioavailability of chrysin was lacking circumstantial clinical evidence in the past years. Nonetheless, recent studies suggested that extensive metabolism by adsorption cells was the factor that caused the oral bioavailability of chrysin was much too low for any biological activity in humans (Galijatovic et al., 1999; Walle et al., 1999; Walle et al., 2001).

Chrysin has also been demonstrated to inhibit the activation of human immunodeficiency virus in models of latent infection (Critchfield et al., 1996; Khoo et al., 2010). Most of these studies focused on the inhibitory activity of reverse transcriptase, or RNA-directed DNA polymerase, but there were other studies on chrysin acted as anti-integrase and anti-protease activities were also described (Nijveldt et al., 2001). However, all of these effects require further clinical validation and verification. An exhaustive line of research shows that chrysin demonstrates anxiolytic properties, which inhibit surgical and non-surgical suppression of Natural Killer (NK) cells activity (Beaumont et al., 2008). NK cells are crucial for defence against infectious diseases and cancers. Several factors have been shown to suppress NK cells activity, including stress, anxiety, surgical procedures and certain anesthetics (Locke et al., 1984; Melamed et al., 2003; Ben-Eliyahu et al., 1999), and hence, surgical suppression of NK cells activity accompanied by prolonged stress may promote metastatic spread of cancers. Therefore, anxiety control and pain management must be an essential element of care and is often accomplished through the use of pharmaceutical agents. This inhibition by chrysin may lead to the suppression of cancer cells metastasis (Beaumont et al., 2008). However, the recent study elucidates the anxiolytic effect of chrysin is blocked by the administration of flumazenil, suggesting that chrysin has a higher possibility to bind to the \( \alpha \)-subunit of \( \gamma \)-aminobutyric acid (GABA) receptor (Dhawan et al., 2002), and hence, further studies are required to elucidate the effects of chrysin on NK cells activity fully under surgical and non-surgical conditions.

### 3 In vitro activities of chrysin

#### 3.1 Chrysin suppresses HIF-1\( \alpha \)/VEGF and angiogenesis

As mentioned in the introduction, chrysin is a natural flavonoid and has been shown recently to have anticancer effects on various cancer cells. However, the molecular mechanisms underlying chrysin on cancer inhibition are not well studied. In this section, investigation showed that chrysin suppresses in vitro expression of Hypoxia-inducible Factor-1 alpha (HIF-1\( \alpha \)) in tumour cells and inhibits in vivo expression of tumour cell-induced angiogenesis through multiple HIF/VEGF pathways, a crucial step in metastasis (Fu et al., 2007; Samarghandian et al., 2011).

In a nutshell, Hypoxia-inducible Factor-1 (HIF-1) is a transcription factor with a heterodimeric structure composed of oxygen regulated \( \alpha \) and ubiquitously expressed \( \beta \) subunits. HIF-1\( \alpha \) is constitutively expressed during hypoxia, but rapidly degraded by the ubiquitin-proteasome pathway in normoxia (Salceda & Caro, 1997; Kallio et al., 1999; Fu et al., 2007). The prolyl hydroxylation of HIF-1\( \alpha \) at the Oxygen-dependent Degradation Domain (ODD) is critical in the regulation of HIF-1\( \alpha \) steady state (Fu et al., 2007). Under hypoxic condition, the absence of oxygen prevents the prolyl hydroxylase from modifying HIF-1\( \alpha \), allowing HIF-1\( \alpha \) to accumulate (Jaakkola et al., 2001, Ivan et al., 2001). Besides, HIF-1\( \alpha \) has been demonstrated to heterodimerize with HIF-1\( \beta \), and this HIF-1 complex acts as a regulator for
more than 70 genes involved in cellular response to reduce oxygen level, thus playing a role in adaptation, survival and progression of tumour cells, including Vascular Endothelial Growth Factor (VEGF) (Miranda et al., 2013) (Figure 2). The intricate interplay between HIF-α isomers in cancer is complicated and yet to be fully deciphered, but the role of HIF-1α activity has been correlated with tumorigenicity and angiogenesis. Many anaerobic human cancers cells are observed to overexpress HIF-1α, because it is induced by hypoxia, cytokines, growth factors, hormones, activated oncogenes and inactivated tumour suppressors (Maxwell et al., 1997; Fukuda et al., 2002; Traxler et al., 2004; Fu et al., 2007; Nagle & Zhou, 2006). Hypoxic tumour cells are more resistant to ionizing radiation and chemotherapy than normoxic tumour cells. These cells are also more invasive and metastatic, resistant to apoptosis and genetically unstable (Melillo et al., 2007). Such areas have been found in a wide range of malignancies: cancers of breast, uterine cervix, vulva, head and neck, prostate, rectum, pancreas, lung, brain tumours, soft tissue sarcomas, non-Hodgkin’s lymphomas, malignant melanomas, metastatic liver tumours and renal cell cancer (Vaupel et al., 2007).

Figure 2: The proposed schematic diagram of the mechanism of HIF-1α and VEGF in angiogenesis. Chrysin is consistently demonstrated to inhibit tumour angiogenesis by targeting multiple HIF-1α/VEGF mechanisms via reducing the HIF-1α stability, dephosphorylating the AKT in the mechanism and reducing the interaction of HIF-1α with Hsp90.

VEGF that is involved in tumour angiogenesis is regulated by HIF-1α in the transcriptional level (Ferrara & Davis-Smyth, 1997; Folkman, 2002; Fu et al., 2007). HIF-1α activates the expression of VEGF gene by binding to the Hypoxia Response Element (HRE) in VEGF promoter (Fang et al., 2005).
In addition to the induction of HIF-1α, other microenvironmental factors are also shown to influence VEGF expression; among them are glucose depletion, glutamine deprivation and acidic extracellular pH (Vaupel et al., 2007). Angiogenesis is critical in tumourigenesis because de novo blood vessel formation must occur to maintain oxygen and nutrient exchange between the tumour periphery and the hypoxic core and metastasis (Folkman 2007). Tumour angiogenesis is stimulated by angiogenic growth factors, such as VEGF, basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor (TGF) and Interleukin-8 (IL-8). VEGF and its receptors have been described as the fundamental regulators of angiogenesis and play an important role in tumour progression (Fang et al., 2005). Therefore, an anti-angiogenic therapy that targets HIF-1α/VEGF system by reducing the HIF-1α level proportionally reducing the expression of VEGF mRNA is a promising strategy for the treatment of human cancers.

The correlation between chrysin and HIF-1α in hypoxia and angiogenesis has been demonstrated and showed that chrysin reduces HIF-1α stability by increasing the prolyl hydroxylation of Oxygen-dependent Domains (ODDs), resulting in an increase in the ubiquitination and proteasome degradation of HIF-1α (Fu et al., 2007) (Figure 2). However, the mechanism of chrysin affecting ODDs remains unknown. A separate study demonstrated that chrysin had the ability also to reduce HIF-1α stability by inhibiting the interaction between HIF-1α and Heat Shock Protein 90 (Hsp90), a chaperone protein (Minet et al., 1999; Isaacs et al., 2002; Katschinski et al., 2002; Nagle & Zhou, 2006). In the studies, the authors suggested that Hsp90 bound to the HIF-1α PAS domains stabilized HIF-1α protein from degradation.

The expression of HIF-1α is regulated not only through protein degradation. It has been reported that growth factors, cytokines and other signalling molecules stimulated also the HIF-1α expression through Phosphatidylinositol 3-kinase (PI3K) (Blancher et al., 2001; Laughner et al., 2001; Stiehl et al., 2002) and target of rapamycin signalling pathways (Treins et al., 2002). Briefly, PI3K/AKT signalling pathway plays an important role in the expression of HIF-1α (Jiang et al., 2001; Blancher et al., 2001). PI3K is a key player in PI3K/AKT signalling pathway, and it is a heterodimeric enzyme composed of 110 kDa catalytic subunit and an 85 kDa regulatory subunit (Carpenter et al., 1990). The best-known downstream target of PI3K is the Serine Threonine Kinase (AKT), which transmits survival signals from growth factors (Chan et al., 1999; Duronio et al., 1998). PI3K/AKT signalling cascade is essential for VEGF expression through HIF-1α response to growth factor stimulation and oncogene activation (Zunde et al., 2000; Blancher et al., 2001; Zhong et al., 2000; Jiang et al., 2001; Fukuda et al., 2002). Conclusively, the role of PI3K/AKT signalling pathway in cancer and angiogenesis is firmly established. Lately, chrysin was found and observed to act as an inhibitor of AKT phosphorylation, and overexpression of active AKT reversed the chrysin-inhibited HIF-1α expression (Fu et al., 2007). Indirectly, this suggests chrysin may inhibit HIF-1α/VEGF-regulated angiogenesis via the AKT signalling pathway (Figure 2). It was also reported that flavonoids or related compounds, such as apigenin (Fang et al., 2005; Osada et al., 2004), resveratrol (Cao et al., 2004), and epigallocatechin-3-gallate (Zhang et al., 2006), inhibited the expression of HIF-1α not only through PI3K/AKT pathway, but multiple signalling pathways. This novel finding provides new insight for chrysin as a potent and versatile inhibitor of angiogenesis and tumourigenesis.

### 3.2 Chrysin downregulates NFκB and its target genes
The anti-cancer potential of chrysin has been further addressed and enhanced by assessing the sensitisation effect of chrysin on Tumour Necrosis Factor-alpha (TNFα)-mediated apoptosis and its related molecular mechanisms (Li *et al*., 2010; Samarghandian *et al*., 2011). This sensitisation effect of chrysin is closely associated with its inhibitory effect on Nuclear Factor kappa B (NFκB) activation, which reduces the expression of NFκB target genes, such as c-FLIP-L that blocking caspase-8 activity (Samarghandian *et al*., 2011) (Figure 3). Generally, NFκB is a transcription factor involved in multiple cellular processes, including apoptosis. Therefore, it is also a target gene for chemopreventive properties of phytochemicals (Li *et al*., 2010; Samarghandian *et al*., 2011). Periodically, NFκB appears as a complex of NFκB:IkB in cytoplasm. The binding of IkB to NFκB prevents NFκB protein to translocate into the nucleus, and hence maintains inactive NFκB in cytoplasm (Hayden & Ghosh, 2004). However, the dynamic balance between cytosolic and nuclear localizations of NFκB is altered upon IkB degradation, resulting in translocation of the activated NFκB into the nucleus where the dimer binds to specific sequences in the promoter or enhancer regions of target genes, such as iNOS, TNFα, IL-1, IL-8, COX-2, CAMs and c-FLIC-L (Ingaramo *et al*., 2013).

**Figure 3:** The proposed schematic diagram of the mechanism of NFκB activation induced by TNFα signalling pathway. Chrysín downregulates NFκB and its target genes whereby enhances the activation of caspase-8 in the apoptotic mechanism.
The sensitisation effect of chrysin on TNFα-induced cell death is achieved through downregulation of NFκB, whereby it reduces of NFκB target gene; cFLIP-L which followed by enhancement of caspase-8 activation. Caspase-8 is the initial caspase in the death receptor signalling pathway that typically induces apoptosis post-ligand treatment. Furthermore, chrysin also shows potential in inducing MAPKp38 and activating NFκB/p65 in cell-cycle arrest and apoptosis (Samarghandian et al., 2011; von Brandenstein et al., 2008).

3.3 Chrys in inactivates PI3K/AKT signalling pathway in apoptosis

Generally, the important effects of chrysin in cellular processes can be concluded to be (1) the inhibition of HIF-1α/VEGF-regulated angiogenesis via the AKT signalling pathway and (2) the sensitisation of TNFα-induced cell death via downregulation of NFκB and activation of caspase-8. In addition, (3) chrysin induces also apoptosis via activation of caspase-3, which involves the inactivation of AKT signalling pathway and downregulation of the X-linked Inhibitor of Apoptosis Protein (XIAP) (Samarghandian et al., 2011). This phenomenon is observed on leukemic cell line (U937) in a previous study that provides the first evidence of a more detailed molecular mechanism on how chrysin induces apoptosis via AKT dephosphorylation in the PI3K signalling pathway. The AKT signalling pathway attracts much attention because of its role in cell survival and the ability to evade cell death pathways in cancer progression. In an overview, the AKT signalling pathway, begins from PI3K to Phosphoinositide-dependent Kinase-1 (PDK1) and end with AKT, mediates apoptosis in human cancer cells. The activation of AKT via phosphorylation prevents apoptosis (Roberts, 2000), whereas dephosphorylation initiates apoptosis. Phosphorylation of AKT phosphorylates Bcl-2-associated Death protein (BAD) and a non-active form of caspase-9, which are the hosts of the cell-signalling proteins. The signalling cascade continues when phosphorylated BAD binds to cytosolic 14-3-3 proteins, resulting in a failure of the protein to heterodimerise with Bcl-2 at the mitochondrial membrane (Kelekar et al., 1997). Dephosphorylation of BAD releases itself from cytosolic 14-3-3 proteins, which subsequently form heterodimers with Bcl-2 family proteins and migrate into the mitochondrial membrane. This is where the heterodimers induce the release of cytochrome c by altering the membrane pores (Pelengaris et al., 2002; Debatin, 2004). Free cytochrome c in the cytoplasm combines with Apoptotic Protease Activating Factor-1 (APAF-1) and caspase-9 to form a complex called apoptosome with the presence of ATP in order to activate the caspase-9 (Debatin, 2004). Subsequently, caspase-9 initiates the downstream executor caspase-3. The activation of caspase-3 follows by degradative events trigger apoptosis (Yoshida et al., 2003; Debatin, 2004). The role of BAD in this molecular mechanism has been investigated. However, the involvement of Bcl-2 Homologous Antagonist/Killer protein (BAK) in this mechanism has not been elucidated in any study previously. It is hypothesized that BAK may have different capacity than BAX to induce apoptosis in cancer cells. Besides, chrysin has also been reported to have the ability to abolish Stem Cell Factor (SCF)/c-Kit signalling by inhibiting the PI3K/AKT pathway (Lee et al., 2007). Monasterio et al. (2004) reported that flavonoids, including chrysin, induced apoptosis via a mechanism that required the activation of caspase-3 and caspase-8, indicating that chrysin-induced apoptosis could operate via a ligand receptor-dependent cell death mechanism. This study suggested also a relationship between AKT and NFκB signalling pathway in the cells. Thus, the study elucidates the relationship between AKT and NFκB with respect to the effects of chrysin in human cancer cells is warranted.
4 Chrysain inhibits proliferation and induces apoptosis in HeLa

4.1 Cervical cancer in Malaysia

Malaysia is a rapidly developing South-East Asian country with an intermediate Gross Domestic Product (GDP) per capita and a significant burden of cervical cancer (Othman & Rebolj, 2009). According to Power Over Cervical Cancer (POCC) in a campaign initiated by the National Cancer Society Malaysia (NCSM), cervical cancer is the third most common cancer among Malaysian females following breast and colorectal cancers. Among the ethnic groups in Malaysia, Indians ranked the highest incidence rate in 2007 at 10.3 per 100,000 persons, followed by Chinese and Malays at 9.5 and 5.3 per 100,000 persons, respectively. On the other hand, the National Cancer Registry (NCR) revealed that the highest number of cancer cases in the country is breast cancer (18.1%), followed by colon cancer (12.3%), lung cancer (10.2%), nasopharynx cancer (5.2%) and cervical cancer (4.6%). On a larger scale, woman dies of cervical cancer every two minutes worldwide. As in other countries in Southeast Asia, the burden of cervical cancer in Malaysia is moderately high. The costs of nationwide spent for cytology-based screening approaches, such as Pap testing that effective in preventing cervical cancer are limited in the region. Moreover, the use of alternative screening modalities, such as visual inspection of the cervix aided by acetic acid (VIA) with or without magnification, is plainly for inspection only. Although prophylactic Human Papillomavirus (HPV) vaccination for the prevention of infection and related disease is considered as an additional cervical cancer control strategy, the service is not accessible to all women in the country. In addition, more efforts should be dedicated to the policy-making context; improve awareness and increase knowledge about cervical cancer using mass media, electronic media, posters and pamphlets to maintain a healthy lifestyle in the community.

Leading a healthy lifestyle has been a way to reduce the risk and the cause of cervical cancer in Malaysia. These efforts include campaigns of consumption more fruits and vegetables, exercising, quitting smoking, taking vitamins, minerals, food supplements and healthcare products. Consuming more fruits and vegetables that contain high levels of flavonoids e.g. chrysain can prevent cervical cancer. Naturally occurring chrysain products are found in the passion flowers Passiflora caerulea and Passiflora incarnata, honeycomb, the mushroom Pleurotus ostreatus, chamomile, Oxylum indicum and Indian trumpet flower. Malaysia has an abundance of traditional medicinal plants and fruits that can be produced for the above-mentioned purposes (Anandhi et al., 2013). As such, complementary and alternative medicine and healthcare products of chrysain should be developed from local traditional medicinal plants and fruits as the primary source of healthcare products or economical in-house regimens for cancer patients in this region.

4.2 In vitro cervical cancer study

Chrysain has been observed to reduce the cytotoxic activity in many human cancer cells. Moreover, the potential apoptotic effect of chrysain has been reported in human cervical cancer, leukemia, esophageal squamous carcinoma, malignant glioma, breast carcinoma, prostate cancer, NSCLC and colorectal cancers (Khoo et al., 2010). Although chrysain has showed to sensitize various human cancer cells to apoptosis substantially (Li et al., 2010), detailed studies of the apoptotic activity of chrysain in cancer cells remain to be elucidated. Furthermore, the apoptotic effect exhibited by chrysain in cervical cancer cells has
not extensively being reviewed with respect to leukemia previously (Table 2). Therefore, chrysin remains its values to be studied for the treatment of human cervical cancer.

Immortal cancer cell lines are used as a useful tool, to facilitate the in vitro screening of novel cytotoxic compounds in human cancer research. Additionally, cell culture facilitates also the study of mechanisms and actions of novel compounds and the structure-activity relationship of the compounds, as well as others in vitro study, for the development of novel anti-cancer agents (Middleton et al., 1994; Lopez-Lazaro et al., 2002). The HeLa cell line is the best known and most widely used human cervical cancer cell line. Indeed, it was the first successful immortal cell line used for the study of human cancers (Masters et al., 2002). Briefly, the HeLa cell line was derived from an adenocarcinoma on the cervix of a 31-year-old black woman, according to the American Type Culture Collection (ATCC). The cells are epithelial, adherent, contain human papillomavirus and react as a suitable transfection host that can be used to

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Reference</th>
<th>Effect and Molecular Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical cancer</td>
<td>Zhang et al. (2004)</td>
<td>Chrysin (IC$_{50}$=14.2 µM) inhibited proliferation and induced apoptosis in HeLa cells, though the effects were not as potent as those of its synthetic derivative compounds.</td>
</tr>
<tr>
<td></td>
<td>von Brandenstein et al. (2008)</td>
<td>Chrysin (30 µM) potentially induced p38 and NFkappaB/p65 activation in HeLa cells.</td>
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<tr>
<td></td>
<td>Lird-prapamongkol et al. (2013)</td>
<td>Chrysin (20-60 µM) sensitized HeLa cells to TRAIL-induced apoptosis by inhibiting STAT3 and downregulating Mcl-1.</td>
</tr>
<tr>
<td>Leukemia</td>
<td>Monasterio et al. (2004)</td>
<td>Chrysin (IC$_{50}$ = 16 µM) showed to be the most potent flavonoid to reduce cell viability and induced apoptotic DNA fragmentation in U937 cells.</td>
</tr>
<tr>
<td></td>
<td>Woo et al. (2004), Woo et al. (2005)</td>
<td>Chrysin induced apoptosis in Bcl-2 overexpressing U937 leukemia cells, was associated with activation of caspase-3 and PLC-γ1 degradation. The induction of apoptosis was accompanied by down-regulation of XIAP and inactivation of AKT.</td>
</tr>
<tr>
<td></td>
<td>Lee et al. (2007)</td>
<td>Chrysin had the ability to abolish SCF/c-Kit signaling by inhibiting the PI3K pathway in myeloid leukemia cells (MO7e).</td>
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<td></td>
<td>Ramos et al. (2008)</td>
<td>Chrysin, alone or in combination with other compounds, decreased AKT phosphorylation and potentially caused mitochondrial dysfunction in THP-1 and HL-60 leukemia cells.</td>
</tr>
</tbody>
</table>

*Table 2: The apoptotic effects of chrysin in leukemia and cervical cancer in vitro (Khoo et al., 2010).*

screen for *Escherichia coli* strains with invasive potential. HeLa cells are indispensable to cancer research, indeed. Therefore, many pharmacological studies and biological evaluations have been carried out using this cell line. For example, several natural and synthetic flavonoids, such as chrysin, are used to determine whether these compounds can inhibit the growth of HeLa cells by inhibiting cell cycle, cell proliferation, oxidative stress, and to induce detoxification enzymes, apoptosis or activate the immune system. Although many efforts have been dedicated to the screening of the preliminary effects of chrysin
using HeLa cells, the exact molecular mechanisms and effects exhibited by chrysin in HeLa cells are not fully understood yet.

One contemporary study showed that the chrysin possibly induced p38 to activate NFκB/p65 in HeLa cells, leading to the apoptosis of the cells (von Brandenstein et al., 2008). Similarly, a few studies also showed that treatment of HeLa cells with 30 µM chrysin for 24 hours induced a significant improvement of NFκB/p65 ranges in the cells, as demonstrated by EMSA. The signals could be suppressed by a specific p38 or p65 inhibitor, indicating that p38 or p65 could be helpful therapeutic target of chrysin in managing gene expression in HeLa cells. More studies are required to determine whether this phenomenon can occur in different manners in HeLa cells, in which NFκB remains the target of research to uncover the mechanisms of apoptosis induced by chrysin in HeLa cells.

4.3 Improving the effects of chrysin in cervical cancer

The biological properties and potential anti-cancer effects of chrysin in human cervical carcinoma (HeLa) can be improved by synthesising diethyl chrysin-7-yl phosphate (CPE: C19H19O7P) and the tetraethyl bis-phosphoric ester of chrysin (CP: C23H28O10P2) through a simplified Antheron Todd reaction (Zhang et al., 2004). The chemical structures indicate the formation of CPE and CP can be achieved by replacing the hydroxyl groups at positions 5 and/or 7 of the A ring in chrysin with phosphate groups (Figure 4). Mass spectroscopy analysis revealed that CPE formed a non-covalent compound with lysozyme, and hence, phosphate esters of chrysin enhanced the interaction of phosphorylated (modified) chrysin with proteins compared to the interaction induced by non-phosphorylated chrysin. The phosphorylated chrysin was concluded to be more effective in inhibiting cancer cell growth and inducing apoptosis in HeLa cells, compared to the original one. This phenomenon can be observed in cultured HeLa cells treated with chrysin, CP and CPE at a concentration of 10 µM for 24 hours, 48 hours and 72 hours. The replacement of both hydroxyl groups at positions 5 and 7 of the A ring in chrysin with phosphate groups proved more effective of the phosphorylated chrysin in inhibiting the growth of cancer cells and inducing apoptosis in HeLa cells more efficiently, compared to the original one. At the same time, this result showed that the cell viability declined in a time-dependent fashion. All chrysin, CPE and CP showed inhibition potency upon proliferation and apoptosis induction in the following order; CP (IC50 = 9.8 µM) > CPE (IC50 = 10.3 µM) > chrysin (IC50 = 14.2 µM) in HeLa cells, using methyl green-pyronin staining and Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) assay, confirming the aforementioned hypothesis. In addition, chrysin, CPE and CP were shown to reduce cell viability by induction of apoptosis and downregulation of the Proliferating Cell Nuclear Antigen (PCNA) in the cells assessed by PCNA immunohistochemistry. Therefore, chrysin and phosphorylated chrysin are suggested as potential potent anti-cancer agents for the treatment of human cervical carcinoma.

5 Perspectives

In conclusion, chrysin inhibits proliferation, induces apoptosis and reduce angiogenesis in most tested cancer cells, including cervical cancer cells. Studies of the mechanisms and actions of the phytochemical reveal that chrysin likely operates by suppressing HIF-1α/VEGF, downregulating NFκB and inactivating PI3K/AKT signalling pathways. However, the inter-relationship of these mechanisms in chrysin-induced apoptosis remains unclear, even though they are known to act via the caspase activation cascade. Other
Bcl-2 family proteins, such as BAK1 and BAK2, might enhance the effect of chrysin-induced apoptosis in HeLa cells. Our current research supports this preliminary hypothesis. However, more studies are warranted. The biological activities of chrysin may be improved by modification of the original structure of chrysin or combination therapy. In addition to structure modification or combination therapy, it is necessary to have chrysin that would not cause general cytotoxicity-related side effects. Although most studies support the conclusion that chrysin induces apoptosis in various tumour cell lines, studies published to date are often haphazardly performed and occasionally contradictory. Hence, more significant research that combine well-designed bioassays and unique sources of chemical diversity are required to understand the exact mechanisms and actions of apoptosis induced by chrysin in human cancers. Results of these studies may help to develop ways of improving the effectiveness of chrysin in the treatment of human cancers.

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