Inhibitory effects and actions of pentacyclic triterpenes upon glycation

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Received 9th of June 2015   Accepted 15th of July 2015
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Keywords: Advanced glycation end-products; Pentacyclic triterpenes; Glyoxalase; Polyol pathway

ABSTRACT

Pentacyclic triterpenic compounds including asiatic, betulinic, maslinic, oleanolic and ursolic acid occur naturally in many herbs and plant foods. It is well known that these triterpenoids possess anti-oxidative and anti-inflammatory activities. Furthermore, recent in vitro and in vivo researches indicated that these compounds could inhibit the production of advanced glycation end-products (AGEs). The impact of these triterpenes upon the activity and protein expression of enzymes involved in polyol pathway including aldose reductase and sorbitol dehydrogenase has been examined, and positive results are reported. These studies suggest that certain triterpenes are potent anti-glycative agents, and may benefit the prevention and/or therapy of glycation-related diseases such as diabetes mellitus and Alzheimer’s disease. In this review article, the anti-glycative activity and action mode of certain triterpenes are highlighted. These information may promote the anti-glycative application of these natural compounds.

1. Introduction

Glycative stress from excessive production of advanced glycation end-products (AGEs) enhances the pathogenic progression of several chronic diseases such as diabetes, aging and Alzheimer’s disease. Several enzymes such as aldose reductase and glyoxalase I are involved in AGEs formation and accumulation. Thus, any agent(s) with the capability to limit AGE generation, promote AGE degradation, and/or reduce the protein expression and activity of associated enzyme(s) may potentially alleviate glycative stress and delay the development of glycation associated diseases.

2. Pentacyclic triterpenes

Triterpenes consist of six isoprene units, and are abundant in the plants in the form of free acids or aglycones [1]. There are more than 80 distinct types of triterpenes have been identified, according to structure and chemical characteristics. Triterpenes are grouped into euphanes, taraxanes, oleananes, lupaneps, ursanes and baccharanes, in which ursanes and oleananes are the major triterpene skeletons in higher plants. In Asian countries including Taiwan, China and Japan, many herbs used for formula or folk medicine are rich in triterpenes and provide medical effects to prevent or treat a variety of diseases [2]. Furthermore, many triterpenes are widely used as edible flavors, pigments, polymers, fibers and glues for food industry or pharmaceuticals. Pentacyclic triterpenes could be further classified into lupane, ursane and oleanane groups. Recently, the bioactivities of certain pentacyclic triterpenoids such as asiatic acid, betulinic acid, corosolic acid, glycyrrhizic acid, maslinic acid, oleanolic acid, ursolic acid, uvaol and erythrodiol have attracted more attention, and been considered as important sources of nutriceuticals and complementary medicines. These pentacyclic triterpenes are present in herbs such as ground ivy (Glechoma hederacea), plantain (Plantago major L.), thyme (Thymus vulgaris), glossy privet (Ligustrum lucidum Fructus), and hawthorn fruit (Crataegi Pinnatifidae Fructus); fresh fruits such as apple (Malus domestica Borkh), carambola (Averrhoa carambola), blueberry (Vaccinium dunalianum), guava (Psidium guajava), calamondin (Citrus microcarpa Bong), persimmon (Diospyros kaki L.), and loquat (Eriobotrya japonica); and fresh vegetables such as olive (Olea europaea L.), gynura (Gynura bicolor DC), daylily (Hemerocallis fulva L.), basil (Ocimum basilicum), water convoeveuhs (Ipomoea aquatica), spinach (Spinacia oleracea L.), mahogany (Toona sinensis) and leaf mustard (Brassica juncea). The content of each pentacyclic triterpene in these edible plant foods is dependent on the species, season, and conditions of cultivation [3-5]. Since the consumption of fresh vegetables and fruits is encouraged for healthy enhancement, more interest has been raised to understand the benefits of these special plant food component(s) upon health and/or disease prevention. Therefore, exploring the bioactivities and action modes of certain pentacyclic triterpenes merits our attention.

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3. Bioactivities of pentacyclic triterpenes

It has been documented that some pentacyclic triterpenes possess anti-oxidative, anti-inflammatory, anti-cancer and vasodilatory activities [6, 7]. These findings suggest that these pentacyclic triterpenes are potent agents for diseases prevention and/or alleviation.

3.1. Anti-oxidative activities

Excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) including superoxide anion, hydrogen peroxide, hydroxyl radical, or nitric oxide has been highly linked to the development and progression of many chronic diseases including aging, diabetes mellitus, cancer, atherosclerosis, Alzheimer’s disease, cirrhosis and Parkinson’s disease [8-10]. It is known that these ROS and RNS, via their free radical property, could directly attack cell apparatus such as mitochondria and impair functions or induce cell death. Furthermore, these ROS and RNS, via acting as signal transduction mediators, could mediate the protein expression of genes encoded for enzymes or factors which are responsible for cell differentiation, growth, migration, invasion and/or apoptosis. These events, in turn, harm the anti-oxidative, anti-inflammatory, anti-cancer and vasodilatory system, enhance oxidative stress and impair invasion and/or apoptosis. These events, in turn, favor the development of acute and chronic diseases. For instance, IL-8 promotes the formation of transforming growth factor (TGF)-beta, which enhances angiogenesis and fibrosis in solid tumors and facilitate cancer metastasis [19, 20]. Thus, the use of appropriate agent(s) with anti-inflammatory effects could decrease the generation of inflammatory stimuli and delay disease progression. The anti-inflammatory effects and possible action modes of several pentacyclic triterpenes in cell lines, animals, and even humans have been reported [21-23]. It is reported that asiatic acid and betulinic acid could regulate nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK), two important signaling pathways, to lower the production of ROS and inflammatory cytokines [24, 25]. Taraxasterol could reduce TNF-alpha, NO and PGE, levels in sera from mice with endotoxic shock [26]. These studies also indicated that these triterpenes were able to regulate both up-stream and down-stream inflammatory factors including COX-2, and inhibit the protein expression of cytokines and/or chemokines. These evidence support that these pentacyclic triterpenes could alleviate inflammatory stress, and decline the progression of chronic diseases.

3.2. Anti-inflammatory activities

Inflammation is a protective process involving host cells, blood vessels, associated proteins and other mediators which are intended to eliminate the invaders and necrotic cells, as well as to repair tissues. Many T-helper cell type 1 and 2 inflammatory cytokines and chemokines including interleukin (IL)-1, IL-6, c-reactive protein (CRP), tumor necrosis factor (TNF)-alpha, cyclooxygenase (COX)-2, monocyte chemotactrant protein (MCP)-1 and prostaglandin E2 (PGE,2), are mediators involved in the host immune and defensive system against stimuli such as pathogens and chemicals [17, 18]. However, under pathological situations such as diabetes mellitus, cardiovascular disease, autoimmune disease and cancer, the overproduction of inflammatory factors causes cytokine imbalance, evokes inflammatory injury, or even leads to tissue destruction. Furthermore, these cytokines and chemokines activate macrophages and/or modulate crucial mediators responsible for pathological processes, which in turn favor the development of acute and chronic diseases. For example, IL-8 promotes the formation of transforming growth factor (TGF)-beta, which enhances angiogenesis and fibrosis in solid tumors and facilitates cancer metastasis [19, 20]. Thus, the use of appropriate agent(s) with anti-inflammatory effects could decrease the generation of inflammatory stimuli and delay disease progression. The anti-inflammatory effects and possible action modes of several pentacyclic triterpenes in cell lines, animals, and even humans have been reported [21-23]. It is reported that asiatic acid and betulinic acid could regulate nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK), two important signaling pathways, to lower the production of ROS and inflammatory cytokines [24, 25]. Taraxasterol could reduce TNF-alpha, NO and PGE, levels in sera from mice with endotoxic shock [26]. These studies also indicated that these triterpenes were able to regulate both up-stream and down-stream inflammatory factors including COX-2, and inhibit the protein expression of cytokines and/or chemokines. These evidence support that these pentacyclic triterpenes could alleviate inflammatory stress, and decline the progression of chronic diseases.

3.3. Other bio-activities

Anti-tumor. Betulinic acid induces mitochondrial membrane permeabilization and causes apoptosis in prostate cancer cells [27]. Maslinic acid executes its cytotoxic activities toward lung cancer A549 cells through mediating mitochondrial intrinsic apoptotic and hypoxia-inducible factor (HIF)-1α pathways [28]. Ursolic acid and oleanolic acid cause apoptosis in liver cancer cells via disturbing mitochondrial membrane ion homeostasis [29]. These findings suggest that these triterpenes are potent anti-cancer agents.

Anti-microorganisms. Asiatic acid and corosolic acid could enhance susceptibility of P. aeruginosa biofilms to tobramycin [30]. Ursolic acid, glycyrrhizic acid and their derivatives may protect liver cells against injury induced by hepatitis B or C virus [31, 32]. Ursolic acid and its derivatives could inhibit the growth of several bacteria including E. coli, S. aureus, P. aeruginosa and K. pneumonia [33]. Apparently, these compounds could be applied for infection prevention or therapy.

Anti-obesity. Ursolic acid may stimulate lipolysis by translocating hormone-sensitive lipase, lowering perilipin A expression, and up-regulating adipose triglyceride lipase in primary culture adipocytes [34]. 18β-Glycyr rhitin acid inhibits adipogenic differentiation and stimulates lipolysis in matured adipocytes [35].

Based on the above reported bioactivities, these pentacyclic triterpenes are potent medicinal agents and could be considered as candidates for new drug development. So far, more information is also available regarding their activities against glycation, another important pathological factor involved in the progression of many chronic diseases.

4. Glycation and chronic diseases

Non-enzymatic glycation with the formation of Maillard reaction products, also known as AGEs, plays an important role in the pathogenesis of many chronic diseases, e.g., diabetes mellitus, Alzheimer’s disease, atherosclerosis, osteoarthritis, inflammatory arthritis and cataracts [36-38]. Any agent(s) with the capability to inhibit the formation of AGEs may potentially diminish glycation stress and delay or retard the progression of glycation-related diseases. It is known that hyperglycemia, a common pathological
characteristic of diabetes, promotes glucose metabolism through the polyol pathway [39]. Aldose reductase (AR), the first and rate-limiting enzyme in this pathway, reduces glucose to sorbitol, which could be further converted to fructose by sorbitol dehydrogenase (SDH), the second enzyme in this pathway [40, 41]. This flux through SDH increases fructose formation, and in turn enhances AGE production and promotes microvascular abnormalities [42, 43]. On the other hand, glyoxalase (GLO)-1, part of the glyoxalase system existed in cytosol of cells, metabolizes reactive alpha-carbonyl compounds such as glyoxal and methylglyoxal, and consequently reduces the available precursors of AGEs [44]. Because AR, SDH and GLO-1 are key enzymes involved in endogenous glycative reactions, and responsible for the generation or degradation of AGEs, the development of new drugs to mediate these pathways and mitigate glycative stress should pay more attention to these enzymes. That is, any agent with the ability to suppress the activity and protein expression of AR and SDH, as well as enhance GLO-1 activity and protein expression, may suppress glycative reactions and decrease AGEs production.

5. AGEs

AGEs are mainly formed by the reactions between reducing sugars including glucose, ribose and ascorbate, and the amino groups of amino acids from proteins or other moieties from lipids or nucleic acids. Amadori rearrangement is involved in AGEs synthesis and leads to various forms and characteristics. That is, AGEs are formed via a complex cascade of reactions including dehydration, condensation, fragmentation, oxidation, and cyclization. In addition, reactive dicarbonyl compounds such as methylglyoxal are precursors, also endogenous source, for the extracellular generation of AGEs [45], because methylglyoxal could easily react with lysine or arginine residues to produce imidazolone adducts, Nε-(carboxyethyl)lysine (CEL) and other compounds [46]. Thus, AGEs are protein-bound mixtures consist of nitrogen- or oxygen-containing heterocyclic compounds. Besides pentosidine and Nε-(carboxymethyl)lysine (CML), the structures and chemical properties of many AGEs need further studies to be characterized. Although lowering AGEs in circulation and tissues could attenuate systemic glycative stress, both decreasing AGE formation and increasing AGE degradation and excretion are difficult challenges because AGEs possess the properties of irreversibly cross-linked, heterogeneous and insoluble protein aggregates. However, the development of anti-AGEs drug is definitely necessary and important in order to control the high incidence and morbidity of glycation associate diseases. Glycated hemoglobin, CML, glycated albumin, and pentosidine may serve as markers of disease progression [47, 48] because they are common AGEs present in the circulation or organs of patients with diabetes mellitus or Alzheimer’s disease. Circulating AGEs are consisted of two sources: exogenous and endogenous. The former is from the dietary intake; the latter are synthesized in the organs such as kidney or heart under normal and pathological conditions. The intake of foods rich in glycative products enriches the circulating AGE pool and also increases local and systemic glycative stress [49]. Obviously, persons with glycation associated diseases should limit their dietary intake of AGE containing foods in order to avoid the deterioration of existed diseases. Endogenous AGEs may be formed during natural aging for most people. Under normal physical condition, the tissue content of AGEs depends on the kinds and rates of AGE formation and degradation. Usually, AGEs deposit in circulation or organs during natural aging is ascribed to the time-dependent property because advanced glycation is always coupled with oxidative stress. That is, AGEs content under this condition could be decreased as long as oxidative stress has been neutralized [50]. However, the progression of diabetes, renal failure, atherosclerosis and neurodegenerative disease markedly promotes endogenous AGE production. It is reported that hyperglycemia or other pathological conditions such as renal failure accelerate the production and accumulation of AGEs locally or systemically, in which oxidative stress plays an important role to boost AGE generation via glycoxidation and lipid peroxidation [51, 52]. On the other hand, AGE degradation is dependent on its ligation to macrophage scavenger receptors, protein turnover rate and renal capability for clearance [53]. Apparently, glycation is regulated by multiple factors and conditions. The management of glycation associate diseases is complicated and attracts more challenge.

6. AGEs in diabetes mellitus

AGEs are effective contributors toward the pathogenesis of diabetes-related macro- and microvascular complications, and the circulating AGEs level are positively correlated to the clinical stage of patients with diabetic complications such as nephropathy or cardiomyopathy [54, 55]. Renal tubular and interstitial cells are the most vulnerable targets for increased glycative stress because hyperglycemia stimulates the tubular cells to secrete vasoactive hormones or factors including angiotensin II, transforming growth factor (TGF)-beta and extracellular matrix proteins such as collagen, which in turn facilitate the synthesis of cross-link components and thicken the basement membrane [56]. These events not only increase AGEs formation in the target cells, but also activate intracellular signal transduction pathways including MAPK and NF-κB, and accelerate the generation of ROS, RNS, inflammatory cytokines, fibrotic factor like fibronectin and angiogenic factors like vascular endothelial growth factor [57, 58]. Finally, an organ or a system is malfunctioned and even failure. In addition, circulating AGEs could be reabsorbed and further metabolized by the proximal tubular epithelial cells. Massive AGEs in renal tissue through declining protein breakdown evoke renal cellular hypertrophy, and cause diabetic nephropathy [59]. After stimulated by AGEs, the organs like kidney could further secrete various intracellular second messengers such as nitric oxide synthase, and subsequently induce the expression of adhesion molecules to benefit the progression of fibrosis and angiogenesis [60, 61]. These studies strongly link AGEs to oxidative, inflammatory and fibrotic injury in diabetes. Therefore, decreasing the level of AGEs in circulation and organs is helpful in order to attenuate or delay the occurrence of these complications.

7. AGEs in Alzheimer’s disease

The brain of patients with Alzheimer’s disease has two major neuro-pathological hallmarks, extracellular senile plaques and intracellular neurofibrillary tangles. Senile plaques contain beta-amyloid (Aβ) peptide, and neurofibrillary tangles contain hyper-phosphorylated microtubule associated protein tau [62]. AGEs could be found in both neurofibrillary tangles and senile plaques. Furthermore, CML is mainly localized in the cytoplasm of neurons, astrocytes and microglia in the brains of elder people
or patients with Alzheimer’s disease [63, 64]. So far, it is still unknown that these AGEs deposited in brain tissue are endogeneously synthesized or exogenously from dietary intake, or may be both. However, it is documented that the accumulation of CML and pentosidine, two major glycative products, are highly associated with the progression of Alzheimer’s disease and other aging associated diseases [65, 66]. These literatures strongly suggest that AGEs play crucial roles for the pathogenesis of brain degradation. AGEs are able to induce neuronal cell apoptosis by acting as neurotoxins or inflammatory mediators to stimulate inflammatory cytokines production and enhance inflammatory stress [67, 68]. Thus, AGEs accumulation in brain neurovascular wall not only changes brain functions, but also speeds up the deterioration of dementia including Alzheimer’s disease. The level of CML and its glycation-specific precursor hexitolysine is markedly increased in neurons of patients with Alzheimer’s disease, especially those with intracellular neurofibrillary pathology [69]. It is commonly considered and accepted that oxidative stress destabilizes many macromolecules including sugars, lipids, proteins and DNA, which promotes glycation reactions and causes an early response in many chronic neurodegenerative diseases such as normal aging process and Alzheimer’s disease. Furthermore, the increased hexitolysine or CML in brain could be partially ascribed to lipid peroxidation in this tissue because of the rich lipid and oxygen content [70], which inevitably leads to neuronal dysfunctions. This oxidative-stress hypothesis regarding the pathogenesis of Alzheimer’s disease suggests that AGES overproduction in brain tissue is accompanied by excessive formation of oxygen-derived free radicals in cerebrovascular disorders [71].

8. Receptors for AGES

The receptor for AGE, also called as RAGE, is expressed in diverse types of cells including endothelial and tubular epithelial cells. RAGEs could engage many ligands associated with distinct pathological processes, which in turn promote the progression of these diseases. AGE is one class of RAGE ligand, and the engagement of AGE with RAGE as occurred in diabetic complications, renal failure or amyloidosis is responsible for glycative stress in these illnesses. So far, enhanced RAGE expression has been reported in various cells under diabetic conditions [72]. It is commonly explained as that hyperglycemia stimulates the production, protein expression, of RAGE ligands, which subsequently interact with AGEs in the circulation and tissues and forms AGE-RAGE complex. Furthermore, AGES, from either dietary source or endogenous source, are able to up-regulate RAGE expression [73]. On the other hand, RAGE acts as a signal transduction receptor for Aβ peptide accumulated in the affected brain parenchyma and cerebral vasculature to enhance the development of Alzheimer’s disease. Thus, the increased AGE-RAGE complex activates signaling pathways, aggravates neuro-inflammation, and finally impairs memory and learning [74]. Meanwhile, RAGE’s ligands are also consisted of $\alpha$100/calgranulins, high-mobility group box 1 and beta-sheet fibrils, and the reactions between RGAE and these ligands generate pro-inflammatory and pro-thrombotic molecules and ROS. These events eventually augment oxidative and inflammatory injury, and thrombotic risk in the target tissues, such as atherosclerotic plaques and cardiac infarction [75, 76]. Besides endothelial and epithelial cells, RAGE and its ligands are also presented in tumor cells, neurons cells, podocytes, and smooth muscle cells. The engagement of RAGE and its ligands may trigger diverse signaling cascades including p21ras, ERK1/2, p38 JNK, and Jak/STAT in these targets [77, 78]. Although RAGEs and their ligands play independent roles in the pathology of several illnesses, AGE-RAGE interaction still attracts more attention because it directly activates many crucial signaling mechanisms. It has been indicated that AGE-RAGE interaction stimulates oxidative stress [79], induces vascular inflammation and thrombosis via activating NF-κB [80, 81], and up-regulates the protein expression of adhesion molecules, chemokines, pro-inflammatory cytokines, matrix metalloproteinases and RAGE itself [82, 83]. Consequently, NF-κB elicits the expression of downstream genes encoded for TNF-alpha, IL-6 and MCP-1, which promote inflammatory reactions and cause irreversible impairment in the target tissues [84, 85]. Glycative stress from AGES, RAGEs and their interaction is a contributor toward the progression of diabetes mellitus and Alzheimer’s disease; however, the impact of this AGE-RAGE axis upon other diseases like cancer could not be ignored. For instance, ROS and cytokines generated from this axis lead to DNA oxidative and inflammatory damage, and may initialize carcinogenesis. Besides AGES and Aβ, RAGE can bind to other ligands such as low-density lipoprotein and calgranulins. Apparently, the impact of AGES, RAGEs, RAGE ligands and their interactions upon human health is not limited to glycate stress. Therefore, any strategy against glycation-associated chronic diseases must consider: (1) lowering the exogenous and endogenous levels of AGES in the circulation; (2) decreasing the available other ligands of RAGE in the circulation; and (3) interfering the interaction of RAGE and its ligands. That is, any possible inhibitor(s) with the capability to block AGE formation, decline RAGE expression, or interrupt the AGE-RAGE interaction could be considered as a potent candidate for treating diabetes mellitus, Alzheimer’s disease, and other glycation-related diseases.

9. Anti-glycative potential of pentacyclic triterpenes

Circulating AGES could be from the dietary intake and from endogenous generation. Many foods are rich in pentosidine and/or furosine, and some of them are processed by sugar, heat or certain sauces [86, 87]. It is reported that foods cooked by baking or deep frying also contain high AGE levels, in which high temperature, lipids and proteins participate Maillard reactions and lead to the synthesis of various forms of AGES [88, 89]. Apparently, in order to decrease circulating levels of AGES from exogenous sources, the consumption of foods rich in glycative products should be limited, especially for patients with diabetes or Alzheimer’s disease. On the other hand, endogenous AGES could be formed between reducing sugars and amino acids present in the circulation and tissues. The pool of reducing sugars and amino acids in the human body is large and unlimited, unfortunately. Thus, lowering the intake of reducing sugars or amino acids may not make any sense for improving health because this limitation definitely impairs nutritional status. The other alternative is to ingest other natural compound(s) with anti-glycative effects to counteract endogenous AGE formation and/or mediate AGE metabolism. Recently, in vitro inhibitory effects of some pentacyclic triterpenes such as astragalosides, boswellic acid and corosolic acid upon the formation of AGES or their precursors like methylglyoxal and CML have been reported [90-92]. The results suggest that these pentacyclic triterpenes may halt the interactions between reducing
sugars and amino acids, decreasing AGE generation and attenuating glycation stress via non-enzymatic actions. Both oxidative and inflammatory reactions benefit glycative processes, and the anti-oxidative and anti-inflammatory activities of several pentacyclic triterpenes such as ursolic acid, oleoanolic acid, corosolic acid, maslinic acid, glycyrrhizic acid and erythroidiol have been already demonstrated in rodents [93-96]. Thus, it is highly possible that these pentacyclic triterpenes directly decrease oxidative and inflammatory stress, which in turn and indirectly mitigates glycative stress. These findings, at least, agreed that these compounds were potent agents and could be considered for anti-glycative protection. The other possibility is to explore the agent(s) with the ability to mediate AR, SDH or GLI, which subsequently reduces the production of AGEs or increases the degradation of AGEs. It is reported that oleoanolic and ursolic acid could inhibit the activity and/or protein expression of AR and SDH, two major enzymes in polyol pathway, which consequently lowered AGEs levels in kidney, liver or brain [97-99]. Furthermore, AGEs metabolism could be facilitated by up-regulating GLO-1. Other studies revealed that protocatechuic acid and glycyrrhizic acid could enhance the expression of GLO-1, and lowered the level of fructose, methylglyoxal and CML in brain of aging mice or kidney of diabetic mice [99, 100]. These studies suggest that endogenous AGE generation could be suppressed by certain pentacyclic triterpenes, which finally contributes to diminish glycative stress under those pathological conditions. The third possibility is to discover the anti-RAGE agent(s). It is strongly convincing that suppressing RAGE expression and/or interrupting the AGE-RAGE interaction could more efficiently block glycative reactions and retard pathological progression in glycation associated diseases. A cell line study indicated that glycyrrhizic acid was able to down-regulate RAGE expression [101]. Ursolic acid could decline RAGE expression in brain of aging mice [102]. The decreased RAGE expression could definitely lower AGES generation and diminish glycative stress. Although the support from in vivo studies regarding the anti-RAGE effects of pentacyclic triterpenes might not be sufficient, the effort toward this direction should be encouraged because the anti-RAGE agent(s) may provide multiple medical benefits. In addition, human studies and clinical trial for these pentacyclic triterpenes regarding their anti-glycative effects and action modes will be highly beneficial to demonstrate their effects and elucidate the actions. Certainly, the exploration of other non-triterpenic anti-glycative agents is also warranted to fight glycation-associated disease.

10. Blood-brain barrier

One crucial challenge of researches regarding pentacyclic triterpenes is their bioavailability. It is a common and important sense that the development of any pharmacological agent against Alzheimer’s disease has to consider whether this substance could pass through the blood-brain barrier, tightly packed layers of endothelial cells, which surrounds the brain to block high-molecular-weight molecules from penetrating it. This blood-brain barrier mainly acts to block the influx of intravascular substances from the circulation to the brain, and also mediates the transport of substances from brain to circulation via several transport systems such as carrier-mediated transport, active efflux transport and receptor-mediated transport [103, 104]. Furthermore, blood-brain barrier is essential for maintaining brain Aβ homeostasis and regulating Aβ transport [105]. Obviously, it is definitely essential to examine whether pentacyclic triterpenes have the capability to pass through the blood-brain barrier.

11. Conclusion

Pentacyclic triterpenes are compounds naturally occurring in many plant foods. Based on their anti-oxidative and anti-inflammatory activities, regulation upon AR, SDH and GLO-1, these agents may improve glycative stress. Future studies should probe the effects and action modes of these pentacyclic triterpenes upon RAGEs, AGE-RAGE interaction and blood-brain barrier penetration. These information could enhance the application of these agents for prevention and attenuation of glycation-associated diseases including, but not limiting, diabetes mellitus and Alzheimer’s disease.

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The development of bioactive peptides from dietary proteins as a dipeptidyl peptidase IV inhibitor for the management of type 2 diabetes

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Received 18th of May 2015 Accepted 25th of June 2015
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Keywords: Antidiabetic effect; DPP-IV inhibitory peptide; In silico; Type 2 diabetes mellitus

ABSTRACT

One of the new approaches to the management of type 2 diabetes mellitus (T2DM) consists of orally administered dipeptidyl peptidase-IV (DPP-IV) inhibitors. These synthetic drug inhibitors are reported to have some side effects and that subsequently limits their applications. There is a growing interest to develop natural DPP-IV inhibitors that will be potent without undesirable side effects. Many in vitro and some in vivo studies have highlighted the potential of food-derived peptides functioning as effective DPP-IV inhibitors. Bioactive peptides within original food-derived proteins are inactive but can be activated by being released during food processing (by enzymatic hydrolysis or fermentation) or during gastrointestinal digestion. Hence, the utilization of computer-aided techniques as screening tools may be helpful in predicting the potential of food proteins as precursors of DPP-IV inhibitory peptides. This paper reviews the current literature on DPP-IV inhibitory peptides, focusing on their in vitro activity and in vivo antidiabetic effects. In addition, the feasibility of various in silico approaches is also summarized in this review.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most prevalent metabolic disorder that is characterized by insulin insensitivity as a result of impaired insulin secretion, insulin resistance, and eventual pancreatic beta-cell failure [1, 2]. T2DM leads to an increase in blood glucose levels [3]. It is reported that 387 million people are living with diabetes mellitus (DM), and, furthermore, DM caused 4.9 million deaths in 2014. It is estimated that by 2035 the number of people affected by DM will reach 592 million with over 90% of them being T2DM [4]. T2DM is a complex disease and is a leading cause of cardiovascular disease, blindness, kidney failure, and lower limb amputation [5]. Therefore, it is important to develop effective strategies to manage T2DM in preventing further progression of this disease and its associated complications.

One of the novel strategies for the treatment of T2DM consists of orally administered dipeptidyl peptidase-IV (DPP-IV; EC 3.4.14.5) inhibitors. The enzyme DPP-IV, a serine protease, has a specificity to remove dipeptides from the N-terminus of substrate poly-peptides by cleaving postproline or alanine residues [6]. It is present in a variety of tissues, particularly epithelial tissues of the liver, kidney and small intestine, and exists as a soluble circulating form [7]. This multifunctional enzyme is implicated in several biological processes, including the degradation of chemokines, neuropeptides, and incretin hormones, e.g. glucose-dependent insulino-inotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [8]. Both the incretin hormones have the potential to stimulate insulin secretion from the islet beta-cell in a glucose-dependent manner [9]. GLP-1 also has some other physiological actions, such as stimulation of insulin biosynthesis, inhibition of glucagon secretion, decrease of gastric emptying and food intake, and enhancement of satiety [10-12]. In normal humans, the incretin effect is mediated mainly by GIP and GLP-1 and is estimated to be responsible for 50-70% of the insulin response to the oral administration of glucose [13, 14]. In T2DM patients, the incretin effect is impaired or absent because of both reductions in the secretion of GLP-1 and pancreatic responses to GIP [13, 15]. In addition, the two incretin hormones have short half-lives of only 1-2 min following their secretion in response to the ingestion of nutrients because of the degradation by the action of DPP-IV [16] that results in the loss of their insulino-inotropic activity [17]. Therefore, the use of DPP-IV inhibitors is a novel approach for the management of T2DM because in using DPP-IV inhibitors the insulinotropic response to GLP-1 is still preserved in patients [18].

A number of DPP-IV inhibitors have been described, many of which have been designed based upon the substrate specificity of the enzyme, potency, oral bioavailability, and duration of action [19]. DPP-IV inhibitors are classified as peptidomimetics and...
non-peptidomimetics. Valine pyrrolidide and isoleucine thiazolidide are the initial peptidomimetic DPP-IV inhibitors that mimic the N-terminal dipeptide as the cleaving site of the enzyme. Vildaglaptin, saxaglaptin, sitaglaptin, and aloglaptin are approved to be antidiabetic agents by the United States and Europe. Although most synthetic DPP-IV inhibitors are generally well-tolerated, some side effects have been recently reported, including nasopharyngitis, headaches, and urinary infections [20, 21]. Therefore, it is important for T2DM therapy to develop a potent DPP-IV inhibitor from natural sources without adverse effects. Dietary proteins, the precursors of a variety of bioactive peptides, have been recognized to improve various aspects of human health [22, 23]. The bioactive peptides embedded within the sequence of a protein can be released by enzymatic hydrolysis, microbial fermentation, and processing methods. A wide range of short-length peptides from dietary protein, e.g. milk [24, 25], rice [26], amaranth, wheat, soybean [27], and fish byproducts [28, 29], have been reported to possess in vitro DPP-IV inhibitory activity. Research on some DPP-IV inhibitory peptides has shown that they are effective at stimulating insulin secretion and improving glycemic control in animal models and subjects with T2DM [30-32]. These effective peptides, having lengths that vary from 3-15 amino acids, particularly involved the presence of at least one proline within the sequence and mostly in the penultimate N-terminal residue [33, 34]. According to the findings in the literature, therefore, developing a tool to assist in the selection of food proteins embedded with DPP-IV inhibitory peptides previously identified is important as well as efficient in predicting the potential of these proteins to manage T2DM.

In recent years, computational (in silico) methods have been demonstrated to be useful in predicting the potential of proteins as precursors of peptides in various bioactivities, such as DPP-IV and angiotensin-I converting enzyme (ACE) inhibitory activities [35-38]. There are two major in silico approaches: the frequency of the occurrence of bioactive peptides within a dietary protein [37], and binding modes by docking analysis [27]. The former is calculated as the number of previously identified bioactive peptides that are found in a given dietary protein; furthermore, the simulation of protein hydrolysis by a bioinformatics tool, e.g. BIOPEP database and program, to find peptides that can be released by a given enzyme is efficient to classify proteins as potential sources of bioactive fragments [36]. The latter, the ligand-enzyme and molecular docking analysis, can simulate the binding and interactions between peptides and enzymes such as DPP-IV and ACE in order to evaluate the inhibitory effects of the peptides [27]. The findings from these two in silico analyses may provide the basis to exploit food proteins as naturally occurring materials for the generation of peptides with DPP-IV inhibitory activity [37]. In the present review, the role and potential of bioactive peptides derived from food proteins to be DPP-IV inhibitors are considered. Future perspectives also receive attention in this review.

2. In vitro DPP-IV inhibitory activity of peptides from dietary proteins

Proteins are well known as precursors of a range of bioactive peptides. The bioactive peptides that are derived from food proteins show a physiological effect in the body in addition to their nutritional values. The fact that proteins are precursors of bioactive peptides is particularly attractive for the development of functional foods because bioactive peptides are commonly used food ingredients and are of natural origins. Food protein-derived peptides can be used as potent alternative pharmaceuticals to chemosynthetic drugs due to an ever-increasing interest in safety and economical usage. The bioactive peptides embedded in their parent proteins are in inactive forms and are activated once released from the proteins by enzymatic or acidic hydrolysis, and their biological activity is determined by their native amino acid composition and sequence [39].

Many DPP-IV inhibitory peptides have been discovered in the enzymatic hydrolysates of various food proteins, including milk proteins [31, 40, 41], rice bran [26], amaranth proteins [27], ham [42], and fish proteins [28, 29]. Table 1 shows a summary of in vitro DPP-IV inhibitory peptides that are ordered by increasing IC₅₀ value as reported in the literature.

Diprotin A, the most potent DPP-IV inhibitory peptide found to date, was isolated from culture filtrates of Bacillus cereus BMF673-RF1 [43]. Diprotin A was produced by reciprocally shaking a culture of the strain BMF673-RF1 for 2 days in a medium containing 1% glucose, 1% glycerol, 1% potato starch, 0.5% polypepton, 0.5% meat extract, 0.5% NaCl, 0.32% CaCO₃, and 0.05% silicon oil KM-70. Diprotin A was identified Ile-Pro-Ile and had an IC₅₀ value of 3.5 μM. A whey protein concentration rich in β-lactoglobulin hydrolyzed by trypsin was fractionated by semi-preparative RP-HPLC [45]. Two (F2 and F3) of the six obtained fractions showed greater DPP-IV inhibitory activities with IC₅₀ values of 367.3 and 86.0 μg/mL, respectively. A peptide, Ile-Pro-Ala-Val-Phe, in fraction F3 was identified as having an IC₅₀ value of 44.7 μM. The peptide Ile-Pro-Ala was also obtained from β-lactoglobulin hydrolysates using proteinase K [41]. This peptide showed one amino acid change in its tis sequence as compared to diprotin A Ile-Pro-Ile (position 3). However, the substitution at position 3 resulted in a weakening of the inhibitory effect versus diprotin A (IC₅₀ value 49 μM versus 3.5 μM). Interestingly, the two peptides, Ile-Pro-Ala-Val-Phe and Ile-Pro-Ala, were both derived from β-lactoglobulin and showed similar DPP-IV inhibitory activity probably due to the same sequences in the first three amino acid residues [41, 45]. A water-soluble extract of a gouda-type cheese ripened for 12 months and then was separated by RP-HPLC [31]. Forty-six peptide sequences contained in the DPP-IV inhibitory fractions from the extract were identified, and two of these peptides were synthesized and showed greater DPP-IV inhibitory activity. Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu and Leu-Pro-Gln were both derived from β-casein and showed greater DPP-IV inhibitory activity. Three peptides, Pro-Gly-Val-Gly-Pro-Leu-Gly-Pro-Ile-Gly-Pro-Cys-Tyr-Glu (1412.7 Da), Cys-Ala-Tyr-Gln-Trp-Gln-Arg-Pro-Val-Asp-Arg-Ile-Arg (1690.8 Da), and Pro-Ala-Cys-Gly-Gly-Phe-Try-Ile-Ser-Gly-Arg-Pro-Gly (1304.6 Da), were identified to show potent DPP-IV inhibitory activities, and their IC₅₀ values ranged from 78 to 116 μM. These peptides have longer lengths than typical DPP-IV inhibitory peptides. The results demonstrate that the DPP-IV inhibitory activity of peptides is determined by the composition and sequence of amino acids rather than their length.

3. In vivo antidiabetic effect of peptides

To date, only a small number of studies have been done on the in
vivo anti-diabetic effects of peptides from dietary proteins. These studies are listed in Table 2. The trypsin-treated β-lactoglobulin was used to evaluate its hypoglycemic efficacy in the C57BL/6 mice model [40]. Mice received the control (0.01 M Tris-HCl buffer), trypsin-treated β-lactoglobulin (300 mg/kg), or sitagliptin phosphate hydrate (3 mg/kg; positive control) by oral administration 1 h prior to an oral glucose tolerance test. The β-lactoglobulin hydrolysate and sitagliptin significantly decreased the blood glucose level at 30 min over the 2-h post-prandial period (P < 0.01), and they both also significantly lowered AUC120min values as compared to the control (P < 0.01). The β-lactoglobulin hydrolysate showed an IC50 value of 210 μM against DPP-IV; and a hexapeptide (Val-Ala-Gly-Thr-Trp-Tyr) isolated from the hydrolysate showed an IC50 value of 174 μM. However, the IC50 value against DPP-IV of sitagliptin phosphate hydrate was 19.6 nM and extremely stronger than the hexapeptide. A peptide, Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu, obtained from a water-soluble extract of gouda-type cheese had an IC50 value of 46 μM against DPP-IV and was used for further evaluation in vivo in rats [31]. The peptide, orally administered to rats at a dose of 300 mg/kg, significantly lowered peripheral plasma glucose concentrations at 30 and 60 min after glucose loading as compared to the control group (P < 0.01). However, the plasma insulin level at each sampling point during the 2-h post-prandial period did not differ significantly between the two groups.

In one study, a zein protein hydrolysate with papain (ZeinH) was found to strongly stimulate GLP-1 secretion in the ileum rather than the duodenum or the jejunum in anesthetized rats [47]. This study also indicated that direct and indirect regulations of GLP-1 secretion mediate not only fat-induced GLP-1 secretion but also dietary peptide-induced GLP-1 secretion in the intestine. Further research was done to evaluate the anti-diabetic effect of ZeinH in rats [48]. The ileal administration of ZeinH (500 mg) significantly decreased the level of glucose in plasma, increased insulin and active GLP-1 concentrations by up to 6.3- and 3.1-folds, respectively, as well as reduced DPP-IV activity by 26.8%, as compared to the control rats (deionized water). In addition, the oral administration of ZeinH (2 or 4 g/kg) showed significantly lower glucose levels in a dose-dependent manner after the glucose injection. The elevation of glucose concentration at 15 min in 4 g/kg ZeinH-treated rats was about half of the concentration that was found in control rats.

The previous research mostly studied the acute treatment of peptides and their effects on some parts of hypoglycemic activity in normal animal models. Ergo, using diabetic animal models to investigate the anti-diabetic effects of peptides may be able to clarify the peptides’ real mechanisms and efficiency. The ZDF (Zucker Diabetic Fatty) rat model of T2DM has been used to

| Peptide sequence | IC50 (μM) | Reference |
|------------------|-----------|-----------|
| Ile-Pro-Ile      | 3.5       | [43]      |
| Ile-Pro-Ile-Gln-Tyr | 35.2     | [38]      |
| Trp-Arg          | 37.8      | [44]      |
| Trp-Lys          | 40.6      | [44]      |
| Gly-Pro-Ala-Gly  | 41.1      | [29]      |
| Gly-Pro-Gly-Ala  | 41.9      | [29]      |
| Trp-Leu          | 43.6      | [44]      |
| Trp-Pro          | 44.5      | [44]      |
| Ile-Pro-Ala-Val-Phe | 44.7      | [45]      |
| Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu | 46 | [31] |
| Ile-Pro-Ala      | 49        | [41]      |
| Cys-Ala-Tyr-Gln-Trp-Gln-Arg-Pro-Val-Asp-Arg-Ile-Arg | 78 | [28] |
| Leu-Pro-Gln      | 82        | [31]      |
| Pro-Ala-Cys-Gly-Gly-Phe-Tyr-Ile-Ser-Gly-Arg-Pro-Gly | 96.4 | [28] |
| Pro-Gly-Val-Gly-Gly-Pro-Leu-Gly-Pro-Ile-Gly-Pro-Cys-Tyr-Glu | 116 | [28] |
| His-Leu          | 143       | [46]      |
| Leu-Pro-Gln-Asn-Ile-Pro-Pro | 160 | [31] |
| Val-Ala          | 168       | [46]      |
| Phe-Pro-Gly-Pro-Ile-Pro-Asp | 260 | [31] |
| Phe-Leu          | 399       | [46]      |
| Ile-Pro          | 410       | [26]      |
| Met-Pro          | 870       | [26]      |
| Val-Pro          | 880       | [26]      |
| Pro-Gly-Pro-Ile-His-Asp-Ser | 1000 | [31] |
| Ile-Pro-Pro-Leu-The-Gln-Thr-Pro-Val | 1300 | [31] |
| Arg-Pro          | 2240      | [26]      |

IC50: half-maximal inhibitory concentration.

| Peptide sequence | IC50 (μM) | Reference |
|------------------|-----------|-----------|
| Ile-Pro-Ile      | 3.5       | [43]      |
| Ile-Pro-Ile-Gln-Tyr | 35.2     | [38]      |
| Trp-Arg          | 37.8      | [44]      |
| Trp-Lys          | 40.6      | [44]      |
| Gly-Pro-Ala-Gly  | 41.1      | [29]      |
| Gly-Pro-Gly-Ala  | 41.9      | [29]      |
| Trp-Leu          | 43.6      | [44]      |
| Trp-Pro          | 44.5      | [44]      |
| Ile-Pro-Ala-Val-Phe | 44.7      | [45]      |
| Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu | 46 | [31] |
| Ile-Pro-Ala      | 49        | [41]      |
| Cys-Ala-Tyr-Gln-Trp-Gln-Arg-Pro-Val-Asp-Arg-Ile-Arg | 78 | [28] |
| Leu-Pro-Gln      | 82        | [31]      |
| Pro-Ala-Cys-Gly-Gly-Phe-Tyr-Ile-Ser-Gly-Arg-Pro-Gly | 96.4 | [28] |
| Pro-Gly-Val-Gly-Gly-Pro-Leu-Gly-Pro-Ile-Gly-Pro-Cys-Tyr-Glu | 116 | [28] |
| His-Leu          | 143       | [46]      |
| Leu-Pro-Gln-Asn-Ile-Pro-Pro | 160 | [31] |
| Val-Ala          | 168       | [46]      |
| Phe-Pro-Gly-Pro-Ile-Pro-Asp | 260 | [31] |
| Phe-Leu          | 399       | [46]      |
| Ile-Pro          | 410       | [26]      |
| Met-Pro          | 870       | [26]      |
| Val-Pro          | 880       | [26]      |
| Pro-Gly-Pro-Ile-His-Asp-Ser | 1000 | [31] |
| Ile-Pro-Pro-Leu-The-Gln-Thr-Pro-Val | 1300 | [31] |
| Arg-Pro          | 2240      | [26]      |

IC50: half-maximal inhibitory concentration.
evaluate the in vivo bioactivity of lysozyme/alcalase hydrolysate in inhibiting DPP-IV [33]. In acute treatment experiments, the hydrolysate and vildagliptin (positive control) were administered by oral gavage of a single dose and were evaluated over a 6 h period. The hydrolysate exerted significant inhibition, approximately 25% inhibition of plasma DPP-IV after 90 min, with a time pattern comparable to that observed after vildagliptin. However, the results of the changes in the associated modulation of metabolic products (glucose, insulin and GLP-1) were not reported. A previous study demonstrated that the peptides (PGH) in the < 1 kDa ultrafiltration fraction of the porcine skin gelatin hydrolysate showed great DPP-IV inhibitory activity and were used for an in vivo animal experiment [32]. A long-term (42 days) in vivo test of streptozotocin (STZ)-induced diabetic rats was used as the animal model to evaluate the anti-diabetic effects of the hydrolysates. Daily administration of PGH (300 mg/day) or sitagliptin (30 mg/day) was able to improve the glucose tolerance in the diabetic rats at days 21 and 42. The DPP-IV activities of the diabetic rats administered PGH and sitagliptin after 42 days were 50.0% and 31.0% lesser than the diabetic control rats, respectively. Furthermore, the PGH and sitagliptin treated rats had an increase of about 10% in active GLP-1 levels and 6-8 fold increase insulin levels as compared to the diabetic control rats. Therefore, the conclusion is that PGH had a superior antidiabetic effect in STZ-induced diabetic rats, including an improvement of glucose tolerance, an elevation of plasma insulin and GLP-1 levels, an inhibition of DPP-IV activity, and a reduction of glucagon levels.

Although there have been a lot of in vitro DPP-IV inhibitory peptides or protein hydrolysates reported, their in vivo effects on diabetic animals or patients have rarely been studied. More detailed in vivo studies to evaluate the efficacy, safety, bioavailability, and potency of inhibitory peptides and/or protein hydrolysates are needed.

4. In silico approaches to predict the potential of peptides as DPP-IV inhibitors

Food proteins are well known to be precursors of bioactive peptides, and these peptides can be released through in vitro hydrolysis by specific enzymes or fermentation by bacteria [50]. The traditional method to screen for the bioactive peptides from a protein involves selecting proteases that have the ability to truncate potent potent peptides according to the literature reports and in vitro experimental tests. However, the key roles of the protein sequence and the specificity of proteases in affecting the generation of bioactive peptides could make this approach costly and time-consuming [35]. Hence, several novel computational approaches to predict the potential of a protein to be the precursor of bioactive peptides by using the combinations of the protein sequences and enzyme specificity have been recently developed [35-38]. The availability of BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep), a database of bioactive peptide sequences, allows for the theoretical prediction of potential bioactivity of different substrates and their corresponding activity after hydrolysis using enzymes with known cleavage specificities [51]. The most commonly used in silico method for . . . predicting the potential of a protein source as a DPP-IV inhibitor is the frequency of occurrence of bioactive fragments in protein sequences. Some researchers have predicted the presence of DPP-IV inhibitors in the sequences of various dietary proteins using the BIOPEP database [25, 37, 38, 52, 53]. They used already known information about the protein sequences and the DPP-IV inhibitory peptide sequences that are currently included in the UniProt Knowledge-base of ExPASy Proteomics Server (http://expasy.org) and BIOPEP database. The potential of each selected protein is quantified based on the frequency of the occurrence of fragments matching peptides with DPP-IV inhibitory activity relative to the length of the protein chain using the following equation [54]:

$$A = a / N$$

where A is the occurrence frequency, a is the number of peptides with DPP-IV activity within the protein chain, and N is the number of amino acid residues in the protein chain.

According to the results of a previous study [37], caseins from cow’s milk and collagens from bovine meat and salmon were found to be the best precursors of DPP-IV inhibitors all with occurrence frequencies over 0.249. Although this kind of in silico approach does provide useful information on the potential of proteins to serve as bioactive peptides precursors, it does not allow the identification of the most potent proteins in terms of inhibitory activity. In consideration of the overlapping sequences and potency (IC₅₀ value) of the DPP-IV inhibitory peptides embedded in protein sequences, two parameters have been established [38]:

- protein coverage (PC) value and potency index (PI).
- The corrected PC value only takes into account the most potent DPP-IV inhibitory peptides.
inhibitory peptide in overlapping areas of the protein sequence. The PI takes into account both the occurrence frequency and potency (IC$_{50}$ value) of the peptides present within a given protein. This study has revealed that the bovine κ-casein is the protein with the highest PI value of 17.89 μM$^{-1}$g$^{-1}$ [38], in particular the potent DPP-IV inhibitory peptide, Ile-Pro-Ile, was found in the sequence. This is in contrast to earlier results that showed that bovine β-casein had the highest occurrence frequency (0.249) of DPP-IV inhibitory peptides, while bovine κ-casein had an occurrence frequency of only 0.130 [37].

Additionally, a docking analysis has also been used as an in silico approach to predict the affinity of the peptides to bind to the active site of DPP-IV. A previous study has shown that the peptide Trp-Trp-Trp had the best docking affinity, was a moderate DPP-IV inhibitor (IC$_{50}$ 216 μM), and its action was non-competitive [Nongonierna et al., 2014]. The authors of the study suggested that the peptide may not bind to the active site of DPP-IV as assumed in the docking prediction. Their results revealed that there is no clear relationship between the docking affinity and the DPP-IV inhibitory activities of the peptides. In addition, they showed that the utilization of molecular docking can be a predictive tool for the competitive inhibitors. Thus, docking can be used as a preliminary tool to help focus experimental screening efforts on a smaller number of candidate peptides.

Since the studies relied entirely on the currently available data on DPP-IV inhibitory peptide sequences, it is possible that other fragments presenting even better DPP-IV inhibitory activity are presently unknown or have not yet been reported in the literature. Furthermore, the putative peptide sequences have to be released from their parent proteins to become active, and the final conclusions on the potential of dietary proteins for DPP-IV inhibitors can be drawn only after experimentally assessing the release of these bioactive peptides upon in vitro or in vivo hydrolysis. Further effort is therefore needed on the development of an in silico approach capable of being used as a screening tool for the evaluation of the potential of dietary proteins for the generation of in vitro and in vivo DPP-IV inhibitors.

5. Conclusions and future perspectives

The importance and scientific understanding of DPP-IV inhibitors that may improve glycemic control in T2DM patients has increased in the last few decades. Much work has been done with food protein-derived DPP-IV inhibitory peptides; however, the evidence of their in vivo antiobdiabetic effect needs to be built in more animal and clinical studies. The “BIOPEP” database is responsible for collecting all of the information about bioactive peptides from academic literature, and 707 protein sequences are responsible for building a knowledge source that is used in more animal and clinical studies. The “BIOPEP” database is available at http://www.idf.org/diabetesatlas/update-2014.

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Protective effects of (-)-epigallocatechin-3-gallate against acetaminophen-induced liver injury in rats

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Received 4th of May 2015  Accepted 30th of June 2015
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1. Introduction

Phytochemicals are found in plant-based foods such as fruits, vegetables, beans, and grains, and they may reduce the risk of a number of chronic diseases including cancer, cardiovascular disease, and diabetes [1]. It is known that phytochemicals can also influence the pharmacological activity of drugs and their toxicities by modifying the drug metabolism system, including drug-metabolizing enzymes and transporters [2, 3]. Acetaminophen (N-acetyl-p-aminophenol, APAP) is an antipyretic and analgesic drug. When an overdose is taken, it can induce severe hepatotoxicity in both humans and experimental animals [4]. APAP is primarily metabolized in the liver by phase II conjugating enzymes, mainly UDP-glucurosyltransferase (UGT) and sulfotransferase (ST), to generate the nontoxic metabolites APAP-glucuronate and APAP-sulfate [4]. The initiation of APAP-induced liver injury results from the cytochrome P450 (CYP)-mediated metabolism of APAP into a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which exerts its toxicity by covalently binding to cellular macromolecules such as proteins, lipids, and DNA [5]. NAPQI also reacts with glutathione (GSH), leading to cellular GSH depletion and the production of reactive oxygen species in the liver. Studies have shown that natural products that decrease CYP enzyme activity, increase antioxidant enzyme activity or GSH levels may attenuate APAP-induced liver toxicity [6, 7].

(-)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin with various biological activities found in tea. In this study, the effects of EGCG on the metabolism and toxicity of acetaminophen in rat liver were investigated. Male Sprague-Dawley rats were fed a controlled diet without or with EGCG (0.54%, w/w) for 1 week and were then intraperitoneally injected with acetaminophen (1 g/kg body weight) and killed after 12 h. Concentrations of acetaminophen and its conjugates in plasma and liver were then determined. The cytochrome P450 (CYP) and phase II enzymes activities were also evaluated. Rats fed the EGCG diet had lower plasma alanine aminotransferase and aspartate aminotransferase activities, as indices of hepatotoxicity, after acetaminophen treatment. Morphological damage by acetaminophen was lower in rats fed the EGCG diet. In addition, EGCG significantly reduced hepatic activities of midazolam 1-hydroxylation (CYP3A), nitrophenol 6-hydroxylase (CYP2E1), UDP-glucosyltransferase, and sulfotransferase. Finally, EGCG feeding reduced acetaminophen-glucuronate and acetaminophen-glutathione contents in plasma and liver. These results indicate that EGCG feeding may reduce the metabolism and toxicity of acetaminophen in rats.

2. Materials and methods

2.1. Materials

Acetaminophen, methoxyresorufin, resorufin, p-nitrophenol, 4-nitrocatechol, NADPH, glutathione, 1-chloro-2,4-dinitrobenzene, and active polyphenol in green tea. Studies suggest that EGCG reduces the development and progress of various diseases such as cancer and cardiovascular disease [8, 9]. The principal hypothesis associated with the putative benefits of tea polyphenols or EGCG is linked to its strong free radical scavenging and antioxidant and anti-inflammatory properties, as well as its modulating effects on drug-metabolizing enzymes, which reduce the bioactivation of carcinogens [10, 11]. Studies have shown that EGCG reduces hepatic CYP3A activity and increases the oral bioavailability of nicardipine and diltiazem in rats [12, 13]. Also, in vitro studies have indicated that EGCG reduces UGT and SUT activities [14, 15]. However, there is currently a lack of information about the effect of EGCG on the phase II detoxifying enzymes in vivo.

Recently, EGCG has been shown to have hepatoprotective activity against chemically induced liver injuries [16, 17]. However, the mechanism of action remains unknown. In this study, we investigated whether EGCG feeding could change the metabolism and toxicity of APAP in rats.

Keywords:
(-)-Epigallocatechin-3-gallate; Acetaminophen; Cytochrome P450; Hepatotoxicity; Rats

ABSTRACT

(-)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin with various biological activities found in tea. In this study, the effects of EGCG on the metabolism and toxicity of acetaminophen in rats were investigated. Male Sprague-Dawley rats were fed a controlled diet without or with EGCG (0.54%, w/w) for 1 week and were then intraperitoneally injected with acetaminophen (1 g/kg body weight) and killed after 12 h. Concentrations of acetaminophen and its conjugates in plasma and liver were then determined. The cytochrome P450 (CYP) and phase II enzymes activities were also evaluated. Rats fed the EGCG diet had lower plasma alanine aminotransferase and aspartate aminotransferase activities, as indices of hepatotoxicity, after acetaminophen treatment. Morphological damage by acetaminophen was lower in rats fed the EGCG diet. In addition, EGCG significantly reduced hepatic activities of midazolam 1-hydroxylation (CYP3A), nitrophenol 6-hydroxylase (CYP2E1), UDP-glucosyltransferase, and sulfotransferase. Finally, EGCG feeding reduced acetaminophen-glucuronate and acetaminophen-glutathione contents in plasma and liver. These results indicate that EGCG feeding may reduce the metabolism and toxicity of acetaminophen in rats.
and heparin, were obtained from Sigma (St. Louis, MO, USA). Midazolam and 1-hydroxy midazolam were purchased from Ultrafine Chemicals (Manchester, UK). All other chemicals and reagents were of analytical grade and were obtained commercially. EGCG was purchased from Huzhou Ruzhou Rongkai Foliage Extract Co. LTD (Huzhou, China). The purity of the EGCG used was >99% as determined by high performance liquid chromatography (HPLC).

2.2. Animal study

First, in the preliminary study, we investigated the effect of EGCG on the drug-metabolizing enzymes in rat livers. Male Sprague-Dawley rats (aged 6 weeks) obtained from BioLASCO in Ilan, Taiwan were used. Rats were fed a laboratory chow diet with or without 0.15% and 0.54% of EGCG for 1 week. Second, to investigate the effect of EGCG on the metabolism and toxicity of APAP, the male Sprague-Dawley rats weighing 210 ± 10 g (6 weeks old) were randomly divided into three groups with six rats in each group. The animals in Group 1 (control group) and 2 were fed a laboratory chow diet. The animals in Group 3 were fed the same diet fortified with 0.54% EGCG. The daily dose of EGCG was about 460 mg/kg in rats, which was equivalent to the dose used in a previous study that found that EGCG did not change the liver function [18]. The rats were all housed in plastic cages in a room kept at 23 ± 1°C with 60 ± 5% relative humidity and a 12-h light-dark cycle. Food and drinking water were available ad libitum for 1 week. At the end of the 1-week feeding period, food was withdrawn for 12 h and a single 1000-mg/kg dose of APAP, as a solution in polyethylene glycol 400/water (50/50, v/v), was intraperitoneally injected into each animal in Group 2 and 3. At 12 h after the APAP dose, the animals in all three groups were killed by exsanguination via the abdominal aorta while under carbon dioxide (70:30, CO2/O2) anesthesia. Heparin was used as the anticoagulant, and the plasma was separated from the blood by centrifugation (1750 × g) at 4°C for 20 min. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were measured immediately by use of commercial kits (Randox Laboratories, Antrum, UK). The liver samples of the three groups were excised and fixed in 10% neutral formalin followed by dehydration in ascending grades of alcohol, clearing in xylene, and embedding in paraffin wax. Liver sections (5 µm thickness) were stained with hematoxylin and eosin (H&E) for the histological examination [19]. The other liver samples from each animal were stored at -80°C. The separated plasma samples and liver homogenates from each animal were used as the probe substrates for methoxyresorufin O-demethylating enzyme activity assays.

2.3. Determination of APAP and APAP conjugates in plasma and liver

Liver homogenates were prepared by homogenizing each gram of liver with 4 mL of ice-cold phosphate-buffered saline (pH 7.4). For determining APAP, APAP-sulfate, and APAP-glucuronate, plasma samples and liver homogenates were diluted 10-fold with control plasma and control liver homogenates, respectively. For determining APAP-glutathione, plasma samples and liver homogenates were not diluted. An aliquot (50 µL) of plasma or liver homogenate was then extracted with 100 µL of acetonitrile and centrifuged at 10,000 × g for 15 min at 4°C. The acetonitrile extract thusly obtained was then analyzed by an HPLC–mass spectrometry (HPLC/MS) method. Calibration standards of APAP, APAP-sulfate, APAP-glucuronate, and APAP-glutathione were prepared by serial dilution of the stock solution of each compound with control plasma or liver homogenate yielding final concentrations of APAP, APAP-sulfate, APAP-glucuronate, or APAP-glutathione that ranged from 1 to 200 µg/mL of plasma or liver homogenate. An aliquot (50 µL) of the spiked plasma or liver homogenate was then extracted with 100 µL of acetonitrile as described above.

To determine hepatic APAP protein adducts, liver homogenate was filtered through a Nanosep centrifugal device (Pall Life Sciences, Ann Arbor, MI, USA) with a membrane molecular weight cutoff of 30 kDa to remove low molecular weight compounds with the potential to interfere in the assay. The filtrate was then digested for 16 hours with proteases to free the APAP-cysteine from APAP protein adducts [7].

The HPLC/MS system consisted of an Agilent 1100 series LC System (Palo Alto, CA, USA). A MightySil RP-18 GP column (5 µm, 250 × 4.6 mm i.d., Kanto Chemical) was used for the determination of APAP. An Agilent Zorbax Eclipse XDB-C18 column (5 µm, 250 × 3.0 mm i.d., Agilent) was used for the determination of APAP conjugates. The HPLC system was interfaced to an Agilent MSD mass spectrometer equipped with an electro-spray ionization source. The column temperature was set to 25 °C. Mobile phase A was 10 mM ammonium acetate containing 0.5% formic acid. Mobile phase B was acetonitrile containing 0.5% formic acid. An isocratic system containing 20%A/80%B was used to determine APAP. The flow rate was 0.5 mL/min. The retention time of APAP was 5.0 min. A gradient system with the following composition was used to determine the APAP conjugates: 90% A (0-2 min), 90% A to 10% A (2-3 min), 10% A (3-5 min), 10% A to 90% A (5-6 min), 90% A (6-12 min). The retention times of the analytes were 2.5 min (APAP-glucuronate), 2.4 min (APAP-sulfate), 2.5 min (APAP-glutathione), and 2.7 min (APAP-cysteine), respectively. The flow rate was 0.5 mL/min. The injection volume was 10 µL. The MS data acquisition was via selected ion monitoring. Ions representing the positive of the testing compound were selected and the peak was measured.

2.4. Preparation of liver microsomes

The frozen liver was thawed and then homogenized (1:4, w/v) in an ice-cold 0.1 M phosphate buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were then centrifuged at 105,000 × g for 60 min. The resulting microsomal pellets were suspended in a 0.25 M sucrose solution containing 1 mM EDTA and were stored at -80°C until they were used. The microsomal protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.5. Drug-metabolizing enzyme activity assays

The CYP enzyme activities were determined by the previously reported HPLC/MS methods [20]. Methoxyresorufin (5 µM), p-nitrophenol (50 µM), and midazolam (2.5 µM) were respectively used as the probe substrates for methoxyresorufin O-demethyla-
Table 1 − Drug-metabolizing enzymes in the liver of rats fed the EGCG diet for 1 week.

|                                      | Control       | EGCG 0.18%    | EGCG 0.54%    |
|--------------------------------------|---------------|---------------|---------------|
| Testosterone 6β-hydroxylase (CYP3A)  | 798.9 ± 43.8  | 599.7 ± 121.6*| 452.2 ± 109.4*|
| p-Nitrophenol 6-hydroxylase (CYP2E1) | 325.5 ± 27.1  | 371.0 ± 34.0  | 357.3 ± 16.6  |
| Methoxyresorufin O-demethylase (CYP1A2) | 31.0 ± 4.5    | 34.0 ± 5.6    | 29.5 ± 4.5    |
| UDP-glucuronosyltransferase          | 53.9 ± 22.2   | 34.0 ± 3.4    | 24.5 ± 4.3*   |
| Sulphotransferase (pmol/min/mg protein) | 1,575.6 ± 47.5 | 1,386.0 ± 26.0* | 1,397.1 ± 38.6* |
| Glutathione S-transferase            | 148.2 ± 4.4   | 108.9 ± 3.5*  | 116.4 ± 10.1* |

Results are expressed as the mean ± S.D. of three rats in each dietary group. *Significantly different from control group, \( P < 0.05 \).

2.6. Determination of GSH and glutathione S-transferase (GST) activity

Liver homogenate was prepared by homogenizing each gram of liver with 10 ml of ice-cold 1.15% KCl and centrifuging the homogenate at 10,000 \( \times g \) for 15 min at 4°C. The resulting supernatant was used to determine the GSH content and GST activity. The GSH content in liver homogenates was determined by HPLC/MS [23]. GST activity was determined spectrophotometrically [24].

2.7. Statistical analysis

Statistical differences among groups were calculated by using a one-way ANOVA (SAS Institute, Cary, NC, USA). The differences were considered to be significant at \( P < 0.05 \) as determined by independent-sample \( t \)-tests.

3. Results

Table 1 shows the effect of EGCG feeding on drug-metabolizing enzyme activity in the liver. Rats fed on the 0.18% and 0.54% EGCG diets for 1 week had significantly reduced \( \text{pmol/min/mg protein} \) of testosterone 6β-hydroxylase (CYP3A) in their livers. In addition, lower ST and GST activities was found in rats that were fed the EGCG diets \( \text{pmol/min/mg protein} \) in their livers. The UGT activity was reduced only in 0.54% EGCG group \( \text{pmol/min/mg protein} \). No significant difference in plasma ALT and AST activities was observed, indicating EGCG caused no hepatotoxicity (data not shown).

After a single dose of the APAP treatment, there were significant differences in body weight and liver weight among the three groups (data not shown). APAP treatment increased plasma ALT and AST activities compared with those same activities in control animals \( \text{pmol/min/mg protein} \). However, rats fed the EGCG diets had lower \( \text{pmol/min/mg protein} \) plasma ALT and AST activities after APAP treatment.

Histological examination of H&E stained liver sections was conducted 12 h after APAP administration to confirm the pattern of hepatotoxicity and compare the extent of liver injury between the control and the EGCG fed animals (Figure 2). Morphological findings were consistent with plasma transaminase observations. The APAP-induced histopathological changes in the liver came with significant degeneration and necrosis of hepatocytes in the centrilobular region and with perivenular inflammatory infiltrates. These APAP-induced histopathological changes were significantly reduced by EGCG treatment. These results indicate that the hepatotoxicity induced by APAP treatment in rats was reduced by EGCG.

Fig. 1 - Effect of EGCG feeding (0.54%, w/w) on plasma alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) in rats after APAP treatment. *Significantly different from control \( P < 0.05 \). #Significantly different from APAP \( P < 0.05 \). Values are the mean ± SD of \( n = 6 \).
APAP-glucuronide, and APAP-glutathione contents in the livers were noted in the EGCG group after APAP treatment. EGCG, however, had no effect on APAP-sulfate and APAP protein adducts contents in rat livers ($P > 0.05$).

The effect of EGCG on drug-metabolizing enzymes after APAP treatment is shown in Table 3. After APAP treatment, there was no significant difference ($P > 0.05$) on the activities of methoxyresorufin O-demethylase (CYP1A2), nitrophenol 6-hydroxylase (CYP2E1), and midazolam 1-hydroxylation (CYP3A); however, GST, UGT, and ST activities were lower than in the control group ($P < 0.05$). Among the APAP-treated groups, CYP3A and CYP2E1 activities were significantly lowered ($P < 0.05$) by EGCG. A significant decrease ($P < 0.05$) in ST activity was observed in rats fed a diet containing EGCG. EGCG had no effect ($P > 0.05$) on UGT activity in rats treated with APAP. In addition, EGCG feeding reversed the reduction of GST activity that was induced by APAP ($P < 0.05$).

A dramatic decrease ($P < 0.05$) in the hepatic GSH level was found in the APAP-treated groups. EGCG had no significant effect ($P > 0.05$) on hepatic GSH content after APAP treatment (Figure 3).

4. Discussion

The results of the present study show that EGCG feeding significantly reduced the elevation of plasma ALT and AST activities that were first induced by APAP. Morphological damage by APAP was lower in rats fed the EGCG diet. In addition, EGCG feeding reduced CYP3A and CYP2E1 activities and lowered APAP-glutathione content in rat livers ($P < 0.05$). Lower ($P < 0.05$) APAP, APAP-glucuronide, and APAP-glutathione contents in the livers were noted in the EGCG group after APAP treatment. EGCG, however, had no effect on APAP-sulfate and APAP protein adducts contents in rat livers ($P > 0.05$).

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### Table 2 – APAP and its related conjugates in the plasma and the liver of the rats.

|          | APAP     | APAP+EGCG |
|----------|----------|-----------|
| **Plasma (μg/ml)**               |          |           |
| APAP     | 371.2 ± 126.2 | 387.5 ± 62.9 |
| APAP-glucuronate       | 135.9 ± 10.0  | 83.5 ± 24.9* |
| APAP-sulfate         | 42.2 ± 3.4    | 36.9 ± 3.0*  |
| APAP-glutathione     | 18.3 ± 2.2    | 10.7 ± 2.9*  |
| **Liver (μg/g liver)**        |          |           |
| APAP     | 116.4 ± 58.1  | 45.9 ± 18.6* |
| APAP-glucuronate| 55.5 ± 3.5    | 32.2 ± 12.4* |
| APAP-sulfate       | 11.0 ± 0.8    | 10.3 ± 0.6   |
| APAP-glutathione   | 58.2 ± 28.4   | 17.6 ± 4.1*  |
| APAP protein adducts| 40.5 ± 3.1    | 39.4 ± 2.7   |

Results are expressed as the mean ± S.D. of six rats in each dietary group. The amount of EGCG in the diet was 0.54% (w/w).

*Significantly different from APAP group, $P < 0.05$. 

After the intraperitoneal injection of a single dose of APAP, plasma APAP concentration remained unchanged in EGCG-treated rats (Table 2). However, EGCG feeding significantly reduced plasma APAP-glucuronide, APAP-sulfate, and APAP-glutathione concentrations in rat livers ($P < 0.05$). Lower ($P < 0.05$) APAP, Table 2 - APAP and its related conjugates in the plasma and the liver of the rats. After the intraperitoneal injection of a single dose of APAP, plasma APAP concentration remained unchanged in EGCG-treated rats (Table 2). However, EGCG feeding significantly reduced plasma APAP-glucuronide, APAP-sulfate, and APAP-glutathione concentrations in rat livers ($P < 0.05$). Lower ($P < 0.05$) APAP, APAP-glucuronide, and APAP-glutathione contents in the livers were noted in the EGCG group after APAP treatment. EGCG, however, had no effect on APAP-sulfate and APAP protein adducts contents in rat livers ($P > 0.05$).

The effect of EGCG on drug-metabolizing enzymes after APAP treatment is shown in Table 3. After APAP treatment, there was no significant difference ($P > 0.05$) on the activities of methoxyresorufin O-demethylase (CYP1A2), nitrophenol 6-hydroxylase (CYP2E1), and midazolam 1-hydroxylation (CYP3A); however, GST, UGT, and ST activities were lower than in the control group ($P < 0.05$). Among the APAP-treated groups, CYP3A and CYP2E1 activities were significantly lowered ($P < 0.05$) by EGCG. A significant decrease ($P < 0.05$) in ST activity was observed in rats fed a diet containing EGCG. EGCG had no effect ($P > 0.05$) on UGT activity in rats treated with APAP. In addition, EGCG feeding reversed the reduction of GST activity that was induced by APAP ($P < 0.05$).

A dramatic decrease ($P < 0.05$) in the hepatic GSH level was found in the APAP-treated groups. EGCG had no significant effect ($P > 0.05$) on hepatic GSH content after APAP treatment (Figure 3).

### 4. Discussion

The results of the present study show that EGCG feeding significantly reduced the elevation of plasma ALT and AST activities that were first induced by APAP. Morphological damage by APAP was lower in rats fed the EGCG diet. In addition, EGCG feeding reduced CYP3A and CYP2E1 activities and lowered APAP-glutathione content in rat livers. These results suggest that EGCG feeding may reduce CYP-mediated APAP bioactivation in liver and, at least in part, contribute to its ability to lower hepatotoxicity.

In addition to lower plasma ALT and AST activities, we also observed that morphological damage by APAP was lower in rats fed the EGCG diet. In this study, hepatic CYP2E1 and CYP3A activities after APAP treatment were reduced in rats fed the EGCG diet. CYP3A and CYP2E1 are two major enzymes that catalyze the oxidative metabolism of APAP and that may generate the toxic
In addition, rats fed tea polyphenols have been shown to have reduced microbial β-glucuronidase activity in the cecum [30]. Therefore, it is possible that EGCG feeding may have reduced enzymatic deconjugation of APAP-glucuronate in the intestine and decreased the re-absorption of APAP via the enterohepatic circulation pathway. The lowered re-absorption of APAP may result in the lower \( P < 0.05 \) APAP content in the liver and thus reduce the CYP-mediated bioactivation of APAP.

In summary, EGCG may act as a hepatoprotective agent against APAP-induced liver injury. Although the exact mechanism is still not clear, our study is the first to demonstrate that feeding rats a diet containing EGCG for 1 week reduces the metabolism and toxicity of APAP.

**Acknowledgments**

This study was financially supported by the grant-aid (NSC 102-2313-B-039-007) of the National Science Council, Taiwan.

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Antibacterial effects and action modes of asiatic acid

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Received 3rd of June 2015   Accepted 20th of July 2015
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1. Introduction

Escherichia coli O157:H7, Salmonella Typhimurium DT104, Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus, Enterococcus faecalis, and Bacillus cereus are seven common foodborne bacterial pathogens [1-4]. These bacteria contaminate many foods including meat, seafood, dairy products, and juice [5-8]. It is well known that contamination from these bacteria reduces the shelf-life of foods, and leads to economic loss for food producers. Most importantly, however, food contamination from these bacteria causes foodborne illness in consumers. The foodborne disease outbreaks due to these bacteria in Taiwan and other countries have been well reported [4, 9]. Therefore, the development and application of a proper agent with antibacterial activity would be helpful in ensuring food safety.

Asiatic acid is a pentacyclic triterpene (Figure 1) that naturally occurs in many vegetables and fruits such as glossy privet fruit (Ligustrum lucidum Ait.), basil (Ocimum basilicum), and brown mustard (Brassica juncea) [10, 11]. It has been reported that this compound exhibits inhibitory effects against S. aureus, B. cereus, E. coli, B. subtilis, and Shigella sonnei, as determined by agar diffusion methods [12, 13]. Garo et al. indicated that asiatic acid could enhance the susceptibility of P. aeruginosa biofilms to tobramycin [14]. The study of Masoko et al. revealed that asiatic acid benefited wound healing via its anti-fungal activity [15]. These previous studies imply that asiatic acid is a potent antimicrobial agent; however, it is unknown whether or not asiatic acid could affect the viability of S. Typhimurium DT104, E. coli O157:H7, L. monocytogenes, P. aeruginosa, and E. faecalis. Furthermore, the minimal inhibitory concentrations (MICs) and action modes of asiatic acid against these bacteria remain unclear.

Abbreviation: CFU, colony forming unit; DMSO, dimethyl sulphoxide; MBC, minimum bactericidal concentration; MH, Mueller Hinton; MIC, minimal inhibitory concentration; OD, optical density.

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Bacterial cell membrane integrity and permeability play crucial roles for bacterial survival and growth. Thus, any agent with the ability to destroy bacterial cell membrane integrity and/or disrupt membrane permeability may cause bacterial cell damage, and even death. In addition, the rupture of bacterial cytoplasmic membrane promotes the release of intracellular components such as potassium ions and nucleotides, which in turn diminishes bacterial ability to repair and replicate [16, 17]. So far, the assays regarding the variation in bacterial membrane integrity, K⁺ efflux, and nucleotides release have been widely used to examine the antibacterial actions of some select agents [18-20]. Thus, if asiatic acid could cause bacterial membrane damage and/or enhance the release of potassium ions and nucleotides, its antibacterial action could be explained.

The major purpose of this study was to investigate the inhibitory effects of asiatic acid against seven foodborne bacterial pathogens. The influence of this compound upon the membrane damage, potassium ions and nucleotides loss in these bacteria was also evaluated. Ground beef was used as a food model to examine the antibacterial effects of asiatic acid at various doses.

### Table 1 – Minimum inhibitory concentration (MIC, μg/ml) and minimum bactericidal concentration (MBC, μg/ml) of asiatic acid against seven bacteria.

| Bacteria                        | MIC  | MBC  |
|--------------------------------|------|------|
| Gram-negative bacteria         |      |      |
| E. coli O157:H7                | 24 ± 4 | 36 ± 4 |
| S. Typhimurium DT104           | 32 ± 2 | 40 ± 4 |
| P. aeruginosa                  | 36 ± 4 | 44 ± 2 |
| Gram-positive bacteria         |      |      |
| L. monocytogenes               | 36 ± 4 | 48 ± 8 |
| S. aureus                      | 28 ± 2 | 44 ± 4 |
| E. faecalis                    | 20 ± 2 | 32 ± 4 |
| B. cereus                      | 40 ± 4 | 52 ± 2 |

Data are expressed as mean ± SD (n = 16).

2. Materials and methods

#### 2.1. Materials

Asiatic acid (98%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Asiatic acid, based on its hydrophobic characteristic, was first dissolved in dimethyl sulphoxide (DMSO, 20 mg/ml), and then used for other preparations. The final concentration of DMSO in the culture medium was maintained at 0.5% (v/v). The impact of DMSO upon the growth of test bacteria was not significant (data not shown).

#### 2.2. Test organisms

Three Gram-negative bacteria, E. coli O157:H7, S. Typhimurium DT104, P. aeruginosa, and 4 Gram-positive bacteria, L. monocytogenes, S. aureus, E. faecalis and B. cereus were recovered from contaminated chicken, duck, and dairy products, as well as seafood from May, 2012 to August, 2013 by using a surface swab technique. Swab samples were directly streaked onto a Chromocult Coliform agar plate, a Brilliant Green agar plate, a Cetrimide agar plate, a Baird Parker agar plate, a Listeria selective agar plate, a brain heart infusion agar plate or B. cereus selective agar plate for E. coli O157:H7, S. Typhimurium DT104, P. aeruginosa, S. aureus, L. monocytogenes, E. faecalis or B. cereus enumeration, respectively. These selective agars were purchased from Oxoid Ltd. (Basingstoke, UK). After sample streaking, agar plates were incubated for 24 h at 37°C. One isolated colony from a contaminated food was defined as 1 isolate. In this study, 16 isolates from 16 different contaminated foods for each test bacterial strain were used for experiments.

#### 2.3. MIC and minimum bactericidal concentration (MBC) determination

MICs of asiatic acid against test bacteria were determined according to the Clinical and Laboratory Standards Institute guideline [21]. Each bacterial strain culture at 0.1 ml, containing 10⁶ CFU/ml as determined by plates count, was inoculated into a 9.9 ml Mueller Hinton (MH) broth (Difco, MI, USA) supplemented with asiatic acid at concentrations ranging from 2 to 512 μg/ml in tubes. All tubes were then inoculated at 35°C for 24 h in an incubator (Model LE-30D, Yih Der Co., Taipei, Taiwan). MIC was recorded as the lowest concentration of asiatic acid to inhibit visible growth of test bacteria, which was reflected by no variation in turbidity. Turbidity was assayed by an optical density (OD) measurement at 600 nm with a UV spectrophotometer (Model UV-1800, Shimadzu Co., Tokyo, Japan). By sub-culturing from the MIC assay tubes onto MH agar plates and incubating at 35°C for another 24 h, MBC was the lowest concentration of asiatic acid to inhibit visible growth on agar plates. All experiments were performed in triplicate.

#### 2.4. Time-kill study assay

*In vitro* time-kill of asiatic acid at 0.5, 1 and 2X MICs (Table 1) against test bacterial strains was monitored in a 10 ml MH broth at 35°C, after inoculation with culture at 10⁶ CFU/ml. At 0, 3, 6, 9 and 12 h, bacterial suspensions at 100 µl were cultured on MH plates for determination of CFU/ml. The plates were incubated at 35°C for 24 h, and the colonies were counted. The detection limit was 20 CFU/ml.

#### 2.5. Heat treatment

A 10 ml beaker with a solution of asiatic acid (20 mg/ml) was sealed with parafilm and placed in a water bath incubator (Model BH-230D, Yih Der Co., Taipei, Taiwan). The temperature of the beakers was maintained at 25, 50, 75 or 100°C for 60 min in this incubator. After cooling down to room temperature, the inhibitory zone of these solutions against the test bacteria was determined.

#### 2.6. Inhibitory zone measurement

The inhibitory zone was determined and compared by disc diffusion method. A sterile blank disc (6 mm diameter, Difco, MI, USA) was soaked in asiatic acid solution for 30 min, and then placed on the surface of a MH agar plate previously seeded with a 100 µl bacterial suspension containing 10⁶ CFU/ml test bacteria. The inhibitory zone was measured after 24 h incubation at 35°C.
2.7. Bacterial membrane damage

A LIVE/DEAD BacLight kit containing SYTO-9 and propidium iodide dyes purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA) was used to measure bacterial membrane damage. Briefly, bacteria were grown in MH broth to an OD_{600} of 0.3, which was equal to 10^{7} CFU/ml determined by plates count, and followed by asiatic acid treatments at 0.5, 1 or 2X MIC for 2 h at 37°C. After centrifugation at 10,000 × g for 15 min, the pellet was collected and resuspended in a buffer containing 5 μM SYTO-9 and 30 μM propidium iodide in the dark for 15 min at room temperature. Green fluorescence, which reflected intact cell membranes, was read at 530 nm; and red fluorescence, which reflected damaged membranes, was read at 645 nm with an excitation wavelength at 485 nm. The ratio of green to red fluorescence intensities determined by a fluorescence microplate reader (Model MTP-601Lab, Hitachi High Technologies, Tokyo, Japan) was normalized to the bacterial sample without asiatic acid treatment.

2.8. Intracellular K⁺ concentration

The K⁺ concentration (ppm) released from the test bacteria was measured by a flame atomic absorption spectrometry (Model 5000, Perkin Elmer Inc., Norwalk, CT, USA), according to the method of Li et al. [22]. Briefly, bacteria were grown in an MH broth to an OD_{600} of 0.3 (10^{7} CFU/ml). One mL of bacterial suspension was treated with asiatic acid at 0, 0.5, 1 or 2X MIC. After 2 h incubation at 37°C, and centrifugation at 10,000 × g for 15 min, supernatants were collected, and the K⁺ concentration was measured.

2.9. Nucleotide leakage

The release of nucleotides was determined by a spectrophotometer (Model U-2000, Hitachi High Technologies, Tokyo, Japan) according to the method of Lou et al. [23]. Briefly, bacteria were grown in an MH broth to an OD_{600} of 0.3 (10^{7} CFU/ml). Asiatic acid at 0.5, 1 or 2X MIC was added into a 1 mL bacterial suspension, and followed by incubating for 2 h at 37°C. After centrifugation at 10,000 × g for 10 min, supernatants were collected, and the absorbance at 260 nm was measured.

2.10. Ground beef preparation

Beer semimembranosus muscle (top round) purchased locally was trimmed of all visible extramuscular fat. The beef muscle was then ground via a 4.5 m/m head on a grinder (Model TS-285, Ta-sin Ltd., Taichung City, Taiwan), and divided into several portions for the following experiments.

2.11. Antibacterial assay in ground beef

Asiatic acid at 0, 2, 4 or 8 mg was mixed with 100 g ground beef. One mL of each test bacterial culture at 10^{4} CFU/ml was added into the 100 g ground beef previously treated with asiatic acid. The inoculated ground beef was then mixed at low speed in a meat mixer (Model TS-383, Ta-sin Ltd., Taichung City, Taiwan) to assure uniform distribution of inoculum. Uninoculated beef samples were also used as negative controls. After days storage at 25°C, 20 g of ground beef was homogenized with 100 mL of deionized water in a Waring blender (Model 31BL91, Sunwei Ltd., Taichung City, Taiwan) at high speed. Then, 1 mL beef homogenate was serially diluted with 9 mL of 0.5% peptone water, and a 0.1 mL portion of each dilution was spread on selective agar plates for enumeration. Plates were incubated at 35°C for 24 h, and colonies were counted and reported as a log of CFU/g ground beef.

2.12. Statistical analysis

All data were expressed as mean ± SD (n = 16). Differences among means were determined by the Least Significance Difference Test with significance defined at P < 0.05.

3. Results

The MICs and MBCs of asiatic acid against the test bacteria are presented in Table 1. MICs were in the range of 20-40 μg/ml; MBCs were in the range of 32-52 μg/ml. The time-kill curves of asiatic acid at 0.5, 1 or 2X MICs against test bacteria strains are presented in Figure 2. After 6 h incubation, the bactericidal effects of asiatic acid upon test strains increased with increasing asiatic acid concentrations from 0.5 to 2 X MIC (P < 0.05). Asiatic acid at 2X MIC effectively reduced bacteria numbers from 6 log_{10} to < 2 log_{10} in all test bacteria within 6 h (P < 0.05). The influence of heat treatments upon the antibacterial effect of asiatic acid, determined by the inhibitory zone, is shown in Table 2. Compared with the 25°C asiatic acid treatment, the 50, 75, or 100°C heat treatments did not significantly affect the antibacterial activity of asiatic acid (P > 0.05).

As shown in Figure 3, asiatic acid dose-dependently impaired membrane integrity (P < 0.05). Asiatic acid at 1 or 2X MICs led to 40-56% and 71-89% membrane damage, respectively, in test bacteria. Asiatic acid treatments also dose-dependently increased bacterial intracellular K⁺ concentrations in test bacteria (Figure 4, P < 0.05). Asiatic acid at 1 or 2X MICs led to 1.5-2.4 ppm and 2.9-4.1 ppm K⁺ release within 2 h, respectively. As shown in Figure 5, asiatic acid treatments caused bacterial nucleotide leakage (P < 0.05). Asiatic acid at 1X MIC increased 1-2.2 folds nucleotide leakage; and at 2X MIC increased 2.7-4.3 folds nucleotide leakage in test bacteria within 2 h. The antibacterial effects of asiatic acid in ground beef are presented in Table 3. After 3 days of storage at 25°C, the addition of asiatic acid dose-dependently inhibited the growth of test bacteria in ground beef (P < 0.05), in which 8 mg of asiatic acid treatments led to bacterial levels in ground beef lower than 2 log_{10}.

4. Discussion

The antibacterial activity of asiatic acid against E. coli, B. subtilis and S. sonneti has been reported before [13]. The results of our present study extend the inhibitory effects of this agent toward other Gram-negative and Gram-positive foodborne bacterial pathogens including S. Typhimurium DT104, P. aeruginosa, L. monocytogenes, S. aureus, E. faecalis, and B. cereus. The MICs of this agent against those bacteria were ≤ 40 μg/ml. Furthermore, we used ground beef as a food model to evaluate the antibacterial potency of asiatic acid. The results reveal that the addition of asiatic acid markedly inhibits bacterial growth in ground beef. These findings indicate that asiatic acid is an effective wide spectrum antibacterial agent against seven foodborne bacterial pathogens in media and ground beef. Thus, this agent could be...
Fig. 2 - Time-kill curves of asiatic acid in broth. Asiatic acid at 0, 0.5, 1 or 2X MIC was added into a Mueller Hinton broth containing $10^6$ CFU/ml bacteria, followed by incubating at 35°C. The level of bacteria was measured at 0, 3, 6, 9 and 12 h. Data are expressed as mean ± SD (n = 16).
Table 2 – Inhibitory zone (mm) of asiatic acid against seven bacteria at 25, 50, 75 and 100°C. The inhibitory zone was determined by disc diffusion method.

| Bacteria                   | 25°C   | 50°C   | 75°C   | 100°C  |
|----------------------------|--------|--------|--------|--------|
| *E. coli* O157:H7          | 33 ± 3*| 31 ± 5*| 32 ± 3*| 30 ± 4*|
| *S. Typhimurium* DT104     | 25 ± 3*| 23 ± 5*| 26 ± 2*| 25 ± 4*|
| *P. aeruginosa*             | 24 ± 5*| 27 ± 4*| 25 ± 3*| 23 ± 2*|
| *L. monocytogenes*          | 28 ± 2*| 26 ± 4*| 30 ± 4*| 27 ± 5*|
| *S. aureus*                 | 30 ± 4*| 33 ± 3*| 29 ± 5*| 32 ± 2*|
| *E. faecalis*               | 35 ± 2*| 36 ± 3*| 38 ± 4*| 35 ± 5*|
| *B. cereus*                 | 22 ± 2*| 20 ± 3*| 23 ± 4*| 21 ± 4*|

Data are expressed as mean ± SD (n = 16).

*Means in a row without a common letter differ, *P* < 0.05.

Fig. 3 - Damage caused by asiatic acid on bacterial membrane. Asiatic acid at 0.5, 1 or 2X MIC was added into a Mueller Hinton broth containing 10^7 CFU/ml bacteria, and incubated for 2 h at 37°C. A BacLight kit was used to determine membrane damage. Data are expressed as mean ± SD (n = 16). **Means among bars without a common letter differ, *P* < 0.05.

Fig. 4 - Effects of asiatic acid on bacterial intracellular K⁺ concentration (ppm). Asiatic acid at 0, 0.5, 1 or 2X MIC was added into a Mueller Hinton broth containing 10^7 CFU/ml bacteria, and incubated for 2 h at 37°C. K⁺ concentration (ppm) was analyzed by a flame atomic absorption spectrometry. Data are expressed as mean ± SD (n = 16). ***Means among bars without a common letter differ, *P* < 0.05.
considered as a potent additive in foods to prevent bacterial contamination. In addition, we found that heat treatments of up to 100°C did not affect the inhibitory effects of asiatic acid against test bacteria. This heat-resistant property benefits its application for foods requiring a heating process of 100°C or lower. That is, this compound is applicable for raw and cold foods, as well as food products treated with high temperature, short time pasteurization.

Bacterial membrane integrity is important not only for bacteria’s self-protection but also for the functions of membrane-associated enzymes responsible for energy generation, respiration, and redox balance [24, 25]. Thus, bacterial membrane rupture easily impairs these critical functions, which in turn affects the bacteria’s survival and growth. In our present study, asiatic acid destroyed the membrane integrity of test bacteria, which subsequently interfered with these above functions, and caused bacterial apoptosis. Potassium ions are the most abundant cations in bacteria such as *E. coli*, and their homeostasis is regulated by K⁺ transporters such as Kdp [26]. These cations are involved in many aspects of bacterial physiological actions including growth, survival, and virulence [27]. Thus, the loss of K⁺ as we observed was definitely detrimental upon the bacteria’s growth and survival. We found that asiatic acid treatments increased K⁺ release from cytoplasm and/or mitochondria in test bacteria. These results indicate that asiatic acid induces irreversible damage of the cytoplasmic membranes, and disturbed K⁺ homeostasis in test bacteria. It is well known that nucleotides such as DNA and RNA are crucial factors responsible for cell repair and replication [28]. The release of nucleotides and their derived compounds including DNA and RNA from bacteria can be quantified by monitoring their absorbance at 260 nm because these substances possess strong UV absorption at this wavelength [29, 30]. In our present study, asiatic acid treatments effectively promoted nucleotides release from the intracellular compartments of test bacteria, which was reflected in their increased absorbance at 260 nm. Since nucleotides were released, the observed death in asiatic acid treated bacteria can be explained. These findings imply that asiatic acid is able penetrate into bacteria, cause damage in DNA-containing organelles like plasma membrane and cytoplasmic membranes, and eventually to cell death.

### Table 3 – Bacterial level (log CFU/g) in 100 g of ground beef treated with asiatic acid at 0, 2, 4 or 8 mg after 3 days of storage at 25°C.

| Bacteria | asiatic acid, 0 | asiatic acid, 2 | asiatic acid, 4 | asiatic acid, 8 |
|----------|----------------|----------------|----------------|----------------|
| *E. coli* O157:H7 | 7.4 ± 0.4⁴ | 4.7 ± 0.2⁵ | 1.6 ± 0.4³ | 0.5 ± 0.3* |
| *S. Typhimurium* DT104 | 8.1 ± 0.5⁴ | 5.2 ± 0.6⁵ | 2.3 ± 0.5³ | 0.7 ± 0.1* |
| *P. aeruginosa* | 7.4 ± 0.6⁴ | 4.4 ± 0.5⁵ | 1.8 ± 0.4³ | 0.4 ± 0.2* |
| *L. monocytogenes* | 7.7 ± 0.5⁴ | 4.5 ± 0.2⁵ | 1.9 ± 0.3³ | 0.3 ± 0.1* |
| *S. aureus* | 8.0 ± 0.7⁴ | 5.3 ± 0.5⁵ | 2.4 ± 0.2³ | 0.4 ± 0.2* |
| *E. faecalis* | 7.5 ± 0.3⁴ | 4.4 ± 0.4⁵ | 1.7 ± 0.6³ | 0.3 ± 0.2* |
| *B. cereus* | 7.8 ± 0.6⁴ | 4.5 ± 0.3⁵ | 2.0 ± 0.4³ | 0.6 ± 0.3* |

Data are expressed as mean ± SD (n = 16). * Means in a row without a common letter differ, *P* < 0.05.

![Fig. 5 - Effects of asiatic acid on bacterial nucleotide leakage. Asiatic acid at 0, 0.5, 1 or 2X MIC was added into a Mueller Hinton broth containing 10⁷ CFU/ml bacteria, and incubated for 2 h at 37°C. The released nucleotide level was determined by measuring absorbance at 260 nm. Data are expressed as mean ± SD (n = 16). **Means among bars without a common letter differ, *P* < 0.05.](image-url)
mitochondria or nuclei, which in turn alters nuclear stability. Our above findings indicate that asiatic acid exerts its antibacterial actions through causing membrane damage, and increasing K⁺ and nucleotides release in test bacteria.

Asiatic acid is a triterpenoid naturally occurring in many edible plant foods [31]. Based on its natural, tasteless, and odorless properties, this agent might be safe and not affect food flavor. In our present study, 8 mg of asiatic acid in 100 g ground beef was equal to 8 ppm, and exhibited markedly anti-bacterial effects. This dosage is not considered high. Thus, the application of asiatic acid to prevent bacterial contamination in foods seems feasible. Our results support the idea that this agent could be applied to foods against bacterial contamination; or used as a bactericide in farms and/or slaughter houses to enhance environmental sanitation. However, further study regarding its safety and possible side effects is necessary before it is used for food preservation. In addition, several animal studies have reported that the dietary intake of asiatic acid could provide anti-diabetic, anti-hyperlipidemic, and hepatic protection via its anti-oxidative activity [32]. It is highly possible that using this triterpene as an antioxidant also improves foods’ oxidative stability and benefits food preservation.

In conclusion, asiatic acid dose-dependently inhibited the growth of E. coli O157:H7, S. Typhimurium DT104, P. aeruginosa, L. monocytogenes, S. aureus, E. faecalis, and B. cereus in medium and in ground beef. Asiatic acid could impair membrane integrity, increase the release of potassium ions and nucleotides in test bacteria. These findings support the contention that asiatic acid is a potent agent to prevent foods from being contaminated by the aforementioned bacteria.

**Conflicts of interest statement**

The authors declare that there are no conflicts of interest.

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The Malnutrition Universal Screening Tool (MUST) and a nutrition education program for high risk cancer patients: strategies to improve dietary intake in cancer patients

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Received 7\textsuperscript{th} of May 2015    Accepted 29\textsuperscript{th} of June 2015
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\textbf{ABSTRACT}

Four hundred and forty-four high-risk oncology patients with malnutrition participated in this study aimed at assessing the effectiveness of nutrition education on improving an oncology patient’s dietary intake. We used a nutritional risk screening to select oncology patients in need of nutritional care. Team Nutrition provides technical assistance for foodservice, nutrition education for patients and their caregivers, and support for healthy eating and physical activity to improve their diets and their lives. The average contribution of protein and total energy of each patient increased after imparting the nutritional education to them. Thus, nutritional education is an effective measure to bring about a favorable and significant change in oncology patients’ nutrient intake.

\textbf{1. Introduction}

The death certification system in Taiwan has been computerized since 1971. Cancer has been the number one cause of death in Taiwan for decades [1]. Cancer-associated malnutrition has many consequences, including increased risk of infection, reduced wound healing, reduced muscle function, and poor skin turgor resulting in skin breakdown [2]. Nutritional support is recommended for malnourished people who are unable to maintain body weight by appetite and food intake often in the decline of a disease. Consequently, tailored strategies to identify patients at nutritional risk are essential to implement nutritional support effectively and to reduce cancer morbidity.

Routine screening for malnutrition should be implemented for people in at-risk groups. The risk of malnutrition and its severity in oncology patients are affected by the tumor type, stage of disease, and the antineoplastic therapy applied [3]. There are many valuable tools that have been developed, validated, and are currently widely used for the detection of malnutrition in clinical practice, including Subjective Global Assessment [4], Mini-Nutritional Assessment (MNA) [5] and its short form (SF-MNA) [6], Nutrition Risk Screening [7] and ‘Malnutrition Universal Screening Tool’ (MUST) [8].

This investigation selected a validated tool that was an easy and simple to screen patients at nutritional risk in oncology, MUST. MUST is a screening tool that has shown its strength for application with adult patients across all healthcare settings including oncology [9]. MUST is a five-step screening tool to identify patients who are malnourished and at risk of malnutrition (or undernutrition). Some strategies can be adopted to improve the nutritional status of these patients. These strategies include patient nutrition education programs, and the use of oral nutritional supplements, which can significantly impact nutritional status [10].

The standard treatment of undernutrition aims to achieve optimal protein and energy intake, according to a patient’s requirements, in order to reduce the effects of catabolism and minimize the loss of the body’s protein mass [11]. The objective of this study is to evaluate if there is a benefit to nutrition education and oral nutritional supplementation on the nutritional status of patients with cancer that are at a high risk of malnutrition.

\textbf{2. Methods}

\textbf{2.1. Subjects}

This is a chart review retrospective cross-sectional observation study that was approved by the Institutional Review Board with...
patients and their families signing waivers of informed consent. Patients were routinely screened with MUST. All cancer patients (n = 444) admitted to the hospital from January, 2011 to December, 2012 who were screened as undernourished (MUST score ≥ 2) at hospital admission were retrospectively included in this study. Patients below the age of 18 years or those who did not complete the nutrition education follow-up were excluded.

This study was conducted at the Chung Shan Medical University Hospital’s (Taichung, Taiwan) cancer care ward. The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects and patient recruitment were approved by Institutional Review Board of the Chung Shan Medical University Hospital Review Board (CSMUH IRB No: CS11124).

2.2. Malnutrition screening tools

The purpose of the MUST system is to detect patients who are at risk for malnutrition or who are malnourished on the basis of knowledge about the association between impaired nutritional status, body composition, and physical function (Figure 1, see www.bapen.org.uk for a free download of tool and an explanatory booklet) [12].

Three independent criteria are used by MUST to determine the overall risk for malnutrition: current weight status using BMI, unintentional weight loss, and acute disease effect that has induced a phase of nil per os for > 5 days. Each parameter can be rated as 0, 1, or 2. Overall risk for malnutrition is established as low (score = 0), medium (score = 1), or high (score ≥ 2). Each of these three criteria can independently predict a clinical outcome, varying by the clinical circumstance, but together the three criteria are better predictors than each by itself [9].

2.3. Study design

The number of MUST scores undertaken by an experienced clinical nurse on patients within 24 h of admission. Data gathered included the numbers of patients with accurately measured height, weight, body mass index, weight loss, and acute disease effect scores. When a MUST score ≥ 2 was calculated, the patient was referred to a dietitian. Step 1 was to give a nutrition assessment and to ascertain from the patient themself, their caregiver(s), and food charts the patient’s past and present appetite and dietary intake, food likes/dislikes, factors affecting nutritional intake, weight history (current, previous, or any weight loss). Then, to go a step further, to formulate a dietetic care plan that involved food selection and meal planning patterns (using oral diet, extra snacks, and possible prescribed supplements). This information was collected before the intervention (baseline) and after the intervention (follow-up) to assess the effectiveness of nutrition education on improving the patient’s dietary intake and nutritional knowledge (Figure 2).
2.4. Data collection and processing

Meal patterns were assessed by asking the caregivers to indicate how many times they provided meals and snacks to the patients. A qualitative 24-hour dietary intake recall was used to determine the adequacy of the patient’s diets. A dietitian assessed all of the foods and drinks that a patient consumed before the nutrition consultation intervention (baseline) and after the intervention (follow-up).

Protein and energy intakes were calculated in grams and kilocalories, respectively, based on the NUFOOD system [13] and the Taiwan food composition table [14]. Data was retrospectively collected using a nutrition care list filled in by a dietitian and discussed with the caregivers. When a patient consumed anything in addition to the hospital menu this was documented precisely by the dietitian.

Other general and medical information, anthropometric data, and information on additional nutrition was obtained from either electronic or written hospital records by using a structured case record form.

2.5. Statistical analysis

The data obtained on food and nutrient intake was then analyzed statistically. Mean and standard error were calculated for each variable. Average daily energy and protein intake by the subjects before and after nutrition education were analyzed by repeated measures ANOVA. McNemar’s test was used to compare the difference in the proportion of energy or protein between the baseline and intervention. Statistical analysis was conducted using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Nutritional parameters

All patients ($n = 444$) were screened using MUST, enabling malnutrition risk. All patients were at risk of malnutrition. 66.4% ($n = 295$) of patients had a BMI $< 20$ kg/m$^2$ (BMI score $\geq 1$). 55.6% ($n = 247$) had unintentional weight loss in 3-6 months $> 5\%$ (weight loss score $\geq 1$). 10.1% ($n = 45$) had an ‘acute disease effect’ NPO $> 5$ days (score $= 2$). Table 1 shows the results of the nutritional parameters using MUST to screen. We also watched the serum albumin level: 44.14% ($n = 196$) of patients had a normal range (Alb $\geq 3.5$ g/dl); 34.46% ($n = 153$) of patients had a mild waste (Alb 2.8-3.5 g/dl); 19.14% ($n = 85$) of patients had a moderate waste (Alb 2.1-2.7 g/dl); and 2.25% ($n = 10$) of patients had a serious waste (Alb $< 2.1$ g/dl). Figure 3 shows the results.
3.2. Protein and Energy Intake

There were 527 cancer patients that MUST screened as high risk. Of those, 83 patients did not finish the follow-up and were excluded from this study. The average daily intake of energy and proteins were 1098.15 ± 539.42 Kcal/D and 0.76 ± 0.41 g/Kg BW respectively before imparting nutritional education. The increase in intake was found statistically significant after imparting the nutritional education, with daily intake of energy and proteins becoming 1578.90 ± 454.74 Kcal/D and 1.16 ± 0.40 g/Kg BW respectively \( (P < 0.0001) \) (Table 2). McNemar’s test shows a statistically significant difference between before and after nutrition education in patients’ energy and protein intake at different levels. Before nutrition education, the daily energy intake was < 500 Kcal 13.96% \( (n = 62) \), 500-1000 Kcal 24.10% \( (n = 107) \), 1001-1500 Kcal 46.85% \( (n = 208) \), and > 1500 Kcal 15.09% \( (n = 67) \). And after nutrition education, the daily energy intake improved to <500 Kcal 2.703% \( (n = 12) \), 500-1000 Kcal 9.459% \( (n = 42) \), 1001-1500 Kcal 29.05% \( (n = 129) \), and > 1500 Kcal 58.78% \( (n = 261) \) \( (P < 0.0001) \) (Table 3). Protein intake before nutrition education was < 0.6 gm/kg 30.41% \( (n = 135) \), 0.6-0.8 gm/kg 34.46% \( (n = 153) \), 0.9-1.2 gm/kg 23.65% \( (n = 105) \), and > 1.2 gm/kg 11.49% \( (n = 51) \). After nutrition education, daily protein intake improved to < 0.6 gm/kg 6.76% \( (n = 30) \), 0.6-0.8 gm/kg 19.37% \( (n = 86) \), 0.9-1.2 gm/kg 27.25% \( (n = 121) \), and > 1.2 gm/kg 46.62% \( (n = 207) \) \( (P < 0.0001) \) (Table 3).

4. Discussion

Malnutrition is common in cancer patients and has a negative impact on disease outcome. Malnutrition increases the duration of the hospital stay [15], reduces the cost-benefit and risk-benefit ratios of anticancer treatments [15], and is directly or indirectly responsible for excess mortality among cancer patients [16]. In an adult the normal range of serum albumin is defined as 3.5-5.0 g/dl and a level < 3.5 g/dl is called hypoalbuminemia [17]. Hypoalbuminemia has been demonstrated to more reliably reflect protein-energy malnutrition than anthropomorphic markers in many studies [18]. There is convincing evidence that the lower the serum albumin level, the higher the risk for postoperative complications and death [19]. Our data show that hypoalbumin-
The guidelines of the European Society for Clinical Nutrition and Metabolism (ESPEN) state that nutritional screening should be able to predict the clinical course based on nutritional status and whether a patient could benefit from nutritional treatment [7]. Screening tools are planned to detect protein and energy malnutrition and/or to predict whether malnutrition is likely to develop or deteriorate under present and future circumstances affecting a patient. In hospitals, further aspects of a disease have to be considered in combination with nutritional measurements in order to determine whether nutritional support is likely to be beneficial. The purpose of the MUST system is to detect adults who are at risk for malnutrition or who are malnourished on the basis of knowledge about the association between impaired nutritional status, body composition, and physical function (Figure 1) [9].

The aim of this study was to investigate whether nutrition education improved protein and energy intakes in undernourished hospitalized cancer patients (Figure 2). The goals of nutritional support in patients with cancer are numerous and include maintaining an acceptable weight and preventing or treating protein-calorie deficiencies, leading to better tolerance of treatment and its side effects, more rapid healing and recovery, reduced risk of infection during treatment, and enhanced overall survival [20]. A systematic review and meta-analysis of oral nutritional interventions in malnourished cancer patients by Baldwin et al. showed that nutritional intervention, including nutritional counseling and oral nutritional supplementation, was associated with statistically significant improvements in weight and energy intake compared with routine care (mean difference in weight = 1.86 kg, 95% CI = 0.25 to 3.47, P = 0.02; and mean difference in energy intake = 432 kcal/d, 95% CI = 172 to 693, P = 0.001) [21].

In this study oral nutritional interventions provided to high-risk cancer patients significantly improved their nutritional status and the quality of the diet consumed, and was associated with statistically significant improvements in protein and energy intake compared with the baseline (mean difference in protein = 0.4 g/d, P < 0.01; and mean difference in energy intake = 480 kcal/d, P < 0.01) (Table 2).

When a patient reported symptoms such as constipation, poor appetite, and abdominal pain, the dietician advised the patient to consume frequent small meals, provided tips for treatment, and provided detailed explanations on food preparation skills to the caregiver in order to increase the nutrition density in food and to prepare a balanced liquid diet. Hutton et al. [22] reported lower energy intake (by 900-1,000 kcal/day), higher rates of weight loss, and lower patient’s quality of life (QOL) scores in patients with severe chemotherapy-associated chemosensory distortions. Cachectic patients should be supplemented with 1000-1500 calories per day (20-25 kcal/kg per day for bedridden patients and 25-30 kcal/kg per day for ambulatory patients) in the form of a balanced essential amino-acid mixture, given between meals [23]. The Recommended Dietary Allowance (RDA) of 0.8 grams (g) of protein per kilogram (kg) of body weight per day is the amount of protein that adequately maintains nitrogen balance in healthy individuals, including the elderly [24]. For optimal dietary supplementation in cachexia, protein source and meal composition also need to be considered, but in practice, the optimal nitrogen supply for cancer patients cannot be determined at present. Protein levels of between 1.2 g and 2.0 g per kg body weight are required to maintain nutritional status according to Johnson [25].

In this study, intervention aimed at a protein and energy intake of 1.2 g/kg and at least 1500 kcal/D respectively per day. Patient’s energy intake went from 1500 kcal/day for 15.09% (67 n) at baseline to 58.78% (261 n) after intervention (p < 0.01), and the protein intake went from 1.2 g/kg/day for 11.49% (51 n) at baseline to 46.62% (207 n) after intervention (p < 0.01) (Table 3).

There are limitations in the implementation of our nutrition education intervention as well as the evaluation instrument that may have influenced the study findings and generalization. Differences in exposure to nutrition information, family support, and food availability and accessibility could influence the patients’ responses to the nutrition education intervention. There may also have been limitations related to the nutrition knowledge, attitude, and food habits as an evaluation instrument. Moreover, this study was limited by the small sample size, with only inpatient participants, and the short duration of study time, which could be considered as being not generalized enough. We suggest further research on a larger sample size and more varieties of participants as well as developing more specific strategies and finding comparative nutrition changes among outpatients.

5. Implications for research and practice

Nutrition intervention in cancer patients can involve many strategies, including dietary counseling and oral nutritional supplementation. Studies concerning the consumption of foods by hospital oncology patients are necessary to establish a relationship between intake values and organic levels, including the checking of the specific nutritional requirements, dealing not only with those on enteral and parenteral diets, but also those on oral hospital diets, who represent the great majority of hospital patients. It is strongly supported that nutritional education can be used as an effective measure to bring about favorable and significant changes in the dietary patterns of hospital oncology patients.

Acknowledgments

This study was supported by a grant from the Chung Shan Medical University Hospital, (CSMUH IRB No: CS11124), Taiwan. We would like to express our sincere appreciation to the subjects for their participation and to all clinical registered dietitians (RD), who kindly provided the supplements for this trial. We also thank the nurses at the cancer care ward for providing expert assistance regarding the Nutritional Screening Project. All other authors declare no conflict of interest.

Conflict of interest

None of the authors reports a conflict of interest.

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Case report

An acute bleeding metastatic spinal tumor from HCC causes an acute onset of cauda equina syndrome

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Received 8th of May 2015    Accepted 26th of June 2015
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1. Introduction

The majority of cauda equina tumors are primary tumors of glial or nerve sheath origin. Indeed, metastases from outside of the central nervous system in this region are very rare [4]. The symptoms of cauda equina lesions are known to be nonspecific. Lower back pain is the most common symptom, followed by sciatic pain, sensory disturbance, motor weakness, and bladder dysfunction [2, 3]. Up to now, only 19 cases, including this case, have been reported in the literature [6]. Herein, we describe the case of a solitary metastatic HCC to the cauda equina with tumor bleeding. To the best of our knowledge, this is the first reported case of an extramedullary-intradural spinal metastasis of hepatocellular carcinoma causing cauda equina syndrome.

2. Case report

A 57-year-old man presented with a 3-week history of worsening lower back pain radiating to the posterior aspect of the bilateral legs. As well, he had an acute onset of bilateral lower leg weakness for 2 days. The patient’s medical history was significant because he had undergone a liver echo guide biopsy that revealed cholangiocarcinoma in August of 2012. Furthermore, he had hepatitis B and liver cirrhosis for many years. During the physical examination, it was noted that there was a significant reduction in the range of motion in the lumbar area due to severe back pain. The pain was worsened by coughing and sneezing. The patient had poor lower muscle strength (Muscle Power: 2/2) and a decreasing sensation to pinpricks over the right thigh (L3). Both plantar responses yielded no response. Patella and Achilles tendon reflexes were diminished in both lower extremities. In addition, he had bladder-urinary dysfunction 2 days before going to the ER. The patient received a laminectomy from the L1 to L4 vertebra, removing the intradural spinal tumor and hematoma. To the best of our knowledge, this is the first reported case of an acute onset of bilateral lower leg weakness as well as bladder-urinary dysfunction 2 days before going to the ER. The patient received a laminectomy from the L1 to L4 vertebra, removing the intradural spinal tumor and hematoma. To the best of our knowledge, this is the first reported case of HCC metastasized to the cauda equina with tumor bleeding causing cauda equina syndrome.

Keywords:
Cauda equina syndrome;
HCC;
Metastatic spinal tumor

ABSTRACT

Hepatocellular carcinoma (HCC) is an aggressive tumor that frequently occurs in the setting of chronic liver disease and cirrhosis. Herein, we describe a case where a patient presented with acute onset cauda equina syndrome due to an intradural and extramedullary metastatic tumor bleeding from hepatocellular carcinoma (HCC). The patient had lower back pain that had radiated to the bilateral lower legs for 3 weeks. Then, the patient had experienced an acute onset of bilateral lower leg weakness and as well as bladder-urinary dysfunction 2 days before going to the ER. The patient received a laminectomy from the L1 to L4 vertebra, removing the intradural spinal tumor and hematoma. To the best of our knowledge, this is the first reported case of HCC metastasized to the cauda equina with tumor bleeding causing cauda equina syndrome.
power of the bilateral lower limbs did not improve and urinary re-
tention still persisted. He underwent radiotherapy for intraspinal 
lesion including T8 and L2/3 level. Because of portal vein inva-
sion and the tumor being larger than 8 cm, the HCC was unresect-
able and TACE was not suitable.

3. Discussion

Hepatocellular carcinoma (HCC) is an aggressive tumor that fre-
quently occurs in the setting of chronic liver disease and cirrhosis. 
The incidence of extrahepatic metastasis reportedly ranges from 
13.5% to 36.7%, and the lungs, adrenal glands, and bone marrow 
are the sites where HCC frequently metastasises [4]. Metastasis 
of HCC to the central nervous system (CNS) is uncommon, and 
this relatively lower incidence of CNS metastasis may be due to 
the low affinity of HCC for the nervous system and the rapid dis-
ease course and short survival time of patients with HCC, which 
decreases the likelihood of the development of CNS metastases 
[5]. The primary lesions that metastasize to the cauda equina 
include the kidney, lung, breast, anus, uterus, ovary, prostate, and 
bone. We are reporting here the first case of extramedullary-in-
tradural spinal metastasis from hepatocellular carcinoma causing 
cauda equina syndrome.

Several routes have been hypothesized by which malignant 
tumour cells might reach the cauda equina [7]: (1) haematog-
enoously via the arterial route, (2) through the extra-intraspinal 
anastomosing venous network (Batson’s plexus) [8], (3) centrip-
etal spread via the perineural spaces [9], (4) by direct extension 
of an extradural mass [10], and (5) further dissemination of CNS 
secondaries along the subarachnoid space as “drop metastases” 
[11]. In our case, the most likely route of dissemination was the 
latter, drop metastases, because the patient had multiple cerebral 
metastases. Perrin et al. [12] reported that 90% of intraspinal me-
tastases are associated with metastatic brain tumors. Intraspinal 
metastasis results when tumor cells descend through the cerebro-
spinal fluid by gravity. Furthermore, occurrence of acute neu-
rological decline after spontaneous tumor hemorrhage was also 
noted in our case.

Treatment options for compressive space-occupying deposits 
have not been clearly defined, and a nonsurgical approach is re-
commended by several authors. Nevertheless, surgical treatment 
of compressive intradural metastases of the cauda equina seems 
to be a feasible treatment option with low operative risk and with 
the potential benefit of an immediate relief of pain and improve-
ment in motor function and thus an increase in quality of life [13].

In conclusion, intradural metastasis to cauda equina is a rare 
ocurrence. Our case presented with bleeding producing further 
neurological deterioration. Hemorrhagic presentation has only 
one been previously reported [14]. Therefore, it is a new factor
that should be considered in differential diagnosis when a patient with a suggestive medical history has the unique pattern of pain suggestive of an intradural tumor. Also, magnetic resonance imaging is a useful tool for detecting intraspinal metastasis.

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