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Effect of Supplementation with *Fructus Lycii* (Kei Tze) on Blood Antioxidant Status: Implications for Age-related Changes in Macular Pigment Density

By

CHENG Chung Yuen

thesis submitted in partial fulfillment of the degree of

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School of Nursing

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Abstract of dissertation entitled:

Effect of Supplementation with Fructus Lycii (Kei Tze) on Blood Antioxidant Status:

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Age-related macular degeneration (AMD) is associated with loss of central vision. It is commonly found in those people over 65 years old. No effective and reliable therapy is available for the treatment of AMD once the vision has been impaired. In terms of preventive eye care, dietary supplementation of food containing high content of lutein and zeaxanthin is believed to be a promising preventive measure for AMD because of the specific accumulation of macular pigment in the macula, which is composed of zeaxanthin and lutein. These carotenoids, particularly zeaxanthin which is concentrated in the central part of the fovea, or yellow spot, may act as a protective filter or an effective antioxidant defense system to meet the continuous oxidative challenge. Scarce research study has been conducted in humans for investigation on the effect of supplementation with Fructus Lycii (Kei Tze) on blood antioxidant or zeaxanthin status. The aims of this study were to investigate the effect of supplementation with Kei Tze on plasma antioxidant status (especially lutein and zeaxanthin). In vitro study of antioxidant content of different Kei Tze products was performed to help guide the choice of supplement used in the in-vivo part supplementation trial. A new High Performance Liquid Chromatography (HPLC) method was set up and evaluated for measuring lutein and zeaxanthin.

Result showed that Kei Tze contains a fairly high antioxidant power 373.5 μmol/g of dried Kei Tze (expressed as Ferric Reducing Antioxidant Power FRAP value), indicating it is
a good source of antioxidants. The new HPLC method for lutein and zeaxanthin is simple, analytically reliable and capable of measuring lutein and zeaxanthin separately and sensitively in human plasma. Precision for plasma lutein and zeaxanthin was good: within-day coefficients of variation (CVs) were 3% at 0.50 µg/ml of lutein (n = 6) and 4% at 0.25 µg/ml of zeaxanthin (n = 6). Between-day coefficients of variation (CVs) were 5% at 0.40 µg/ml of lutein (n = 6) and 6% at 0.20 µg/ml of zeaxanthin (n = 6). Recovery of lutein and zeaxanthin were good with overall recovery of at least 90% for both. The linearity was maintained to at least 2.0 µg/ml for lutein and to a least 1.0 µg/ml for zeaxanthin (r = 0.999). The minimum detectable limit for lutein and zeaxanthin was found to be 0.3 ng. The concentration of lutein and zeaxanthin in Kei Tze used in our supplementation study was 5.8 µg/g and 194 µg/g of Kei Tze respectively. This provides a good reference for comparison with other research data as scarce scientific data are available for consumer, scientist or healthcare professionals.

In the in-vivo study of Kei Tze, we conducted a single-blinded, placebo-controlled, parallel intervention study for 28 days. Fasting blood, urine and saliva were collected from 27 apparently healthy subjects (study group n = 14, placebo group n = 13) at day 0 for baseline. Repeated fasting samples were collected for the study group and the placebo group at day 28 after supplementation of 15 g/day of dried Kei Tze and placebo respectively. Results showed that 28 days supplementation of Kei Tze maintained the baseline plasma antioxidant capacity (expressed as FRAP) and uric acid in the study group after the supplementation study. No significant change was found for urine FRAP and saliva FRAP before and after the supplementation. However, it is of interest that the plasma FRAP values and uric acid were significantly (p<0.05) lower in the placebo group compared to the treatment group after 28 days. A significant increase (p<0.01) in plasma zeaxanthin was found after Kei Tze supplementation. This has not been previously reported in the literature. No statistically
significant changes were seen in any of other variables measured, such as plasma ascorbic acid, lutein, superoxide dismutase (SOD), glutathione peroxidase (GPx) and urine hydrogen peroxide (H₂O₂), however the pattern of change was interesting. Whole blood GPx concentration tended to increase and urine hydrogen peroxide tended to decrease in the study group after 28 days Kei Tze supplementation.

These new data indicate that Kei Tze intake can maintain or replenish the plasma antioxidant capacity. No deleterious effect on measured variables was seen. The data in our supplementation study demonstrated Kei Tze causes a significant increase in plasma zeaxanthin after 28 days supplementation. Hopefully, this increase in plasma zeaxanthin may help to prevent AMD by maintaining the macular pigment density, however this remains to be confirmed in future study.
Acknowledgement

I would like to express my sincere thanks to Dr. I.F.F. Benzie, Professor of the School of Nursing, Hong Kong Polytechnic University (HKPU) for her excellent guidance and supervision throughout the course of my work. I am very grateful to her help in finding the sponsor of the wolfberry used in the supplementation trial. I would also like to thank Dr. Y.T. Szeto, Research Fellow of the School of Nursing, Hong Kong Polytechnic University (HKPU) for his technical support and valuable advice on my laboratory work. My thanks go to Dr. W.Y. Chung, who gave me a lot of his professional advice on the setting up of High Performance Liquid Chromatography (HPLC). I would also like to thank Kenwick Industries Limited for the sponsor of Rich Nature™ Wolfberry used in this supplementation study. Last but not least, I thank all members in the antioxidant research team, the School of Nursing, Hong Kong Polytechnic University (HKPU) for their generous supports in my supplementation trial.
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<td>H₂O₂</td>
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<td>NaOH</td>
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<td>PDT</td>
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<td>PUFA</td>
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<td>RPE</td>
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<td>SARS</td>
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<tr>
<td>TC</td>
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1. Literature Review

1.1 Introduction

In most developed countries, incidence of age-related macular degeneration (AMD) is rising annually among the elderly. The impact of the socio-economic burden and of the visual impairment on their quality of life cannot be neglected. To our disappointment, no effective treatment is available for these patients. Though some innovative ideas have been recently proposed for the potential treatment of AMD, these remain at very early stages of trial. Not only the lack of desirable treatment but also the mystery of the potential risk factors hampers the progress of research in age-related maculopathy. Even the plausible hypothesis that oxidative stress can result in AMD suffers from, inconsistent findings among different studies, and much further work is needed to resolve the real picture of AMD, its risk factors, underlying mechanism and pathology.

It is strongly believed that antioxidant defense mechanisms in our body are critical to fight against the continuous oxidative attack in our eyes (Rose et al, 1998). It is further believed that oxidative damage to the macula is central to AMD, and that increasing intake of certain antioxidants found in high levels within the normal macula may prevent AMD. This is a very important aspect in this virtually untreatable condition. Thus, use of dietary supplements and a well-rounded diet seems to be highly focused for preventive measure. The question is, what foods or supplements would be most effective in helping to prevent or delay onset of AMD.

In traditional Chinese medicine, it is believed that the small red berry of the plant *Fructus Lycii* (′Wolfberry′; ′Kei Tze′) is good for vision because of its high content of antioxidants. However, its potential application for prevention of AMD has been rarely evaluated. With the increasing use of Chinese herbs in Western medicine, it is seen to be a promising area for dietary supplementation for maintenance of macular health. This is the
focus of this study.

1.2 Age–related maculopathy

AMD is one of the leading causes of blindness among people over 65, and yet its aetiology remains poorly understood. Owing to its unknown aetiology, significant clinical impact of knowledge in relation to this on patients, and its unlimited potential for research in ophthalmology, it is undoubtedly an important topic in biomedical research. Compared with other well-known eye diseases such as glaucoma and cataract, AMD is a 'hidden' disorder and most people are not familiar with AMD. However, this "vision killer" is very common among the elder in this decade. With global population ageing, AMD is an eye disease causing high concern among health professionals. Without a definitive cause of AMD, many researchers focus on this challenging topic, aiming at exploring its mystery. However, studies are complicated by its late onset, clinical heterogeneity and complex aetiology. Furthermore, studies of AMD are sometimes hampered by the conflicting results among different studies. Currently, no primary prevention or desirable medical treatment is available for AMD. That is why most clinicians can only monitor the decline in vision after diagnosis of AMD, adopting a conservative approach in the clinical management of patients until the condition is well advanced, after which laser treatment may be offered (Stokkermans, 2000b). Without more effective treatment a marked decline or loss of central vision is almost unavoidable. Eventually, this produces a significant impact on the quality of life. Hence, it is an undeniable fact that improved understanding of AMD is a milestone to contribute to the development of treatment and hopefully even prevention of this disease.
1.3 Pathology of AMD

With a view to giving a basic understanding on AMD, concise pathological changes will be firstly reviewed. To obtain a clear image in our vision, the surface of the retina should be smooth and flat. Otherwise, it will lead to blurring of the image. For a normal eyeball, the macula is a critical area which contains the highest concentration of photoreceptors, and is situated in the central part of the retina (York et al, 2000). The retina lies on a flat carpet of blood vessels. At the interface between the retina and the blood vessels, there is a compact layer called Bruch’s membrane. In order for nutrients to be supplied to the very actively metabolizing retina, nutrients must be transported from the blood vessels to the retinal layer through the Bruch’s membrane (Figure 1).

![Figure 1](image)

**Figure 1** Cross sectional drawing of the eye, with enlargement (insert) showing a healthy retina (York J et al, 2000)
As people get older, cumulative damage occurs in many body sites, including the eye, and there is good evidence that oxidative damage is responsible for ageing and increased risk of age-related diseases, such as AMD. The retina consumes more oxygen than any other tissue and is richly supplied with blood. Reactive oxygen intermediates such as hydrogen peroxide and superoxide are continuously produced as byproducts of oxygen metabolism. In addition, the retina is particularly susceptible to oxidative stress because the combination of photosensitizers/light/O₂/polyunsaturated fatty acids (PUFA) found in the retina results in activated oxygen intermediates, such as singlet oxygen, and lipid peroxidation by-products which can cause cytotoxic oxidative damage (Winkler et al, 1999). The outer segments of the photoreceptors cells contain PUFA and this microenvironment is likely to promote the formation of more free radical in response to radiation (sunlight) exposure, yielding highly oxidized lipid materials which are not easily digested by phagocytosis in the retinal pigment epithelial (RPE) cells (Algvere et al, 2002). Normally, the RPE cells are responsible for photoreceptor renewal, and are essential for normal retinal function. The shed photoreceptor discs are digested in the phagosomes of RPE cells (Bernstein et al, 1999). It takes about 10 days to renew an entire human rod outer segment by this disc-shedding process. However, in aged RPE cells, the digestion of phagosomes in the lysosomes is incomplete, resulting in residual products such as lipofuscin. These noxious, undigested products from the RPE cells will gradually accumulate on the inner collagenous layer of Bruch’s membrane, forming characteristic yellow mounds which are called drusen (Figure 2).
In fact, the formation of drusen is a continuous "physiological" process which is evident between 20 and 70 years of age. Most often, the concentration of lipofuscin reaches the peak concentration at the macular area. However, this drusenoid material is associated with several adverse effects on the RPE cells function and survival. At a certain critical point nutrient flow will be blocked by continuous accumulation of drusen. Finally, the eye will respond in one of the following ways, either dry or wet AMD. In dry form, which account for about 90% of AMD cases, the retinal cells eventually waste away due to shortage of nourishment (Figure 3). Thus, the vision becomes blurred.

Figure 2 The formation of lipofuscin in aged RPE cells. In aged RPE cells, the phagocytosis of photoreceptor outer segments eventually insufficient. The digestion of phagosomes in the lysosomes is incomplete, yielding residual products such as lipofuscin, which is noxious to RPE cell function. Undegraded products from RPE cells, including outer segment material, accumulate on the inner collagenous layer of Bruch’s membrane. (Algvere PV et al, 2002)
Figure 3  Cross sectional drawing of retina with dry macular degeneration. (York et al, 2000)

Figure 4  Cross sectional drawing of retina with wet macular degeneration. (York et al, 2000)
In wet AMD, choroidal neovascularization (CNV) is typically found. In a recent animal study, it was found that over-expression of vascular endothelial growth factor (VEGF) in the RPE can lead to the development of CNV (Spilsbury et al, 2000). That means new feeder blood vessels will grow into the thickened Bruch’s membrane as the eye attempts to improve nutrient flow. However, these abnormal feeder vessels are fragile and may leak fluid or blood under the retina. Exudates accumulate at the Bruch’s membrane and the macular surface becomes elevated, which finally lead to blurred vision (Figure 4). Hence, wet AMD is also called exudative AMD. In severe form of wet AMD, it will cause rapid loss of central vision.

1.4 Clinical manifestation

At the early stage of AMD, small drusen and pigmentary abnormalities are seen below the retinal layer. Initially, vision can be good at this stage. As the disease progress, these patients will present with reduced visual acuity, blurred vision and reading difficulties. Hence, elderly individuals over 65 years of age are suspected to have AMD when they presenting with these clinical symptoms. In reality, most of the elderly are not well educated for this eye disease. Therefore, they are not alert to and may even ignore these warning signs because they may think that their poor vision is due to ageing only. Without any medical consultation, the condition will eventually evolve into late stage of AMD. In dry AMD, it advances so slowly that people hardly notice any difference. With progressive macular degeneration, clinical findings of dry AMD are characterized by the presence of drusen and geographic atrophy of the retinal pigment epithelium. That is why dry AMD is also called atrophic AMD. In wet AMD, it progresses at a much faster pace. Generally, typical signs of wet AMD include CNV, lipid exudation, dirty-gray oedema, haemorrhagic detachment of RPE and disc-forming scarring (Campochiaro et al, 1999). Worse yet, the damage is beyond
repair. In most new cases, the patients suffer from a certain degree of vision impairment during their first visit to the ophthalmologist. Hence, more strenuous effort should be paid on educating the elderly to increase their awareness on this eye disease.

1.5 Epidemiology

In the past, different population-based epidemiological studies have been performed (Klein et al, 1999a). However, the estimated prevalence of AMD is quite variable (Hawkins et al, 1999; Klein et al, 1999b). These variable results are due to the lack of agreement on the definitions of AMD. Apart from these, the methods used to detect and classify AMD may vary from study to study. Hence, comparisons of findings become difficult. To make more comparable epidemiological data sets, an international classification system for AMD has been proposed (Bird et al, 1995). Therefore, it will be expected that more conclusive results would be found soon. Among Caucasian, there appears to be little geographic variation in the prevalence of signs of late AMD. The overall prevalence of late AMD in the Beaver Dam population was 1.7% (Klein et al, 1992). Exudative macular degeneration was present in at least one eye in 1.2% of the population, and geographic atrophy in 0.5%. These estimates are very similar to those found in the Rotterdam (1.2%) (Vingerling et al, 1995), Blue Mountains (1.4%) (Mitchell et al, 1995) and Framingham Eye Studies (1.5%) (Kahn et al, 1977) in persons less than 86 years of age. Focusing in Hong Kong, the number of patients suffering from AMD seems to be increasing rapidly. However, little informative statistical data has been published. Only little work has been done concerning the prevalence of AMD in Chinese. In the Hong Kong Vision Study (Van Newkirk, 1997), it was found that age-related maculopathy was observed in 5.9% of the participants. As the definition of age-related maculopathy in this vision study included both early AMD (including the presence of soft or reticular drusen and RPE abnormalities) and late AMD (including geographic atrophy of RPE
and neovascular complications of AMD), this explained why the levels of Chinese subjects seem much higher than the Western population because previous studies mainly focused on late AMD only. For AMD, it accounted for 19% of the moderate visual impairment in this study, which can be ranked as second leading cause of visual disability in elders. With this figure, it is obvious that the prevalence of AMD cannot be neglected in Hong Kong.

1.6 Risk factors

In hopes of reducing this disease, the importance of identifying different risk factors associated with AMD cannot be neglected, especially those that can be modified by either personal or medical interventions. Basically, risk factors for AMD can be categorized into non-modifiable and modifiable (Hyman et al, 2002). Factors such as age, sex, race and genetic factors are classified as non-modifiable. Others are modifiable factors such as cigarette smoking, body mass index, obesity, diet habits, including dietary fat intake and possibly, alcohol consumption, and sunlight exposure.

For non-modifiable risk factor, age is the strongest risk factor for prevalence and incidence of age-related maculopathy. Data from the Beaver Dam Eye Study has identified that features of AMD increase in frequency with advanced age. Focusing on gender, AMD prevalence has been found to be higher in women in some but not all studies, thus leaving the question of increased AMD risk in women unsettled (Smith et al, 2001). Apart from these, several findings suggested that late stages of AMD are more common among whites than blacks (Schachat et al, 1995; Friedman et al, 1999). One possible reason for the lower frequency of exudative AMD among those with darker pigmentation is that increased melanin in RPE cells may act as a free radical scavenger and can protect the RPE from oxidative damage. However, other studies showed conflicting results, which make ethnicity as a risk factor remain questionable. Besides, it was suggested that AMD could be a
multifactorial disorder, which has different environmental and genetic causes. Nowadays, extensive evidence implicates the substantial role of genetic factors in AMD. For example, several familial aggregation studies showed a strong correlation of AMD in first degree relatives (Seddon et al, 1997). Also, twin studies revealed that in monozygotic twins the concordance of AMD is often 100% (Meyer et al, 1988; Meyer et al, 1995) again supporting a role for genetic make-up in AMD. Even with this supporting evidence, the major counterstone is that the exact gene loci accounting for AMD susceptibility is still unknown. Allikmets found that some heterozygous ABCR gene mutations can predispose to AMD (Allikmets et al, 1997). However, after the publication of these findings, it drew a lot of criticism from other researchers (Dryja et al, 1998). The arguments focused on the lack of grading system for patient’s selection, insufficient racial matching and inappropriate data interpretation etc. In the following few years, many researches focused on hunting for a causative gene in AMD (Klein et al, 1998; Weeks et al, 2000; Shroyer et al, 1999). However, many results have been subjected to controversy (De La Paz et al, 1999; Sachiko et al, 1999; Nobuo et al, 2000; Eric et al, 2000). Apart from ABCR gene, the apolipoprotein E (ApoE) gene has been suggested to be a susceptibility gene for AMD. However, different studies gave conflicting findings (Souied et al, 1998; Klaver et al, 1998; Pang et al, 2000; Leung et al, 1999; Silke et al, 2000). The elucidation of the genetic basis of AMD is one of the most challenging in ophthalmic research within the next decade.

Focusing on the modifiable risk factors, cigarette smoking is the only risk factor other than age that has been consistently identified in numerous studies (Smith et al, 1996), although the exact mechanism for this association remains unclear. It has been shown that risk of AMD increases with increasing number of cigarettes smoked per day. Not only the number of cigarettes smoked but also the risk of early and exudative AMD was higher for current versus former smokers (Christen et al, 1996). Besides, it was found that body mass
index and obesity were risk factors for dry AMD but not for neovascular AMD (Schaumberg et al, 2001). However, variability of findings across different studies means that the strength and nature of this association remain unclear. Recently, it was suggested that higher intake of specific types of fats, including vegetable monounsaturated and polyunsaturated fats and linoleic acid rather than total fats, were positively associated with neovascular AMD (Seddon et al, 2001). Although the role of dietary fats in AMD is still doubtful, this risk factor remains to be determined by further research study. For alcohol consumption, no consistent findings across studies supported an association between alcohol consumption and AMD (Cho et al, 2000). Therefore, it seems to be unlikely to be associated with AMD. For sunlight exposure, it was suggested that exposure to bright sunlight may cause changes in the RPE similar to changes seen in AMD. However, no conclusive finding was observed in animal and human studies (Delcourt et al, 2001) and the association between sunlight exposure and AMD remains doubtful. Therefore, it is obvious that most of the above risk factors have been inadequately investigated and there is insufficient evidence to establish conclusive associations. It is an undeniable fact that prevention is better than cure. Thus, more extensive studies should be paid in order to explore the underlying basis of different risk factors on AMD, thus allowing us to modify our lifestyles for prevention of this eye disease.

1.7 Diagnostic techniques

Without any effective treatment of AMD, early detection of clinical symptoms becomes the key to success for combating AMD. Nowadays, different conventional techniques have been used for diagnosis. Clinically, commonly used techniques include Amsler grid testing, indirect ophthalmoscopy, fundus photography, fundus fluorescein angiography (FFA) (Gutner et al, 1997). Each test has its own limitations, so the clinician should choose carefully. For example, Amlser grid testing (Figure 5) is the most preliminary,
qualitative method for assessing central vision. This functional test is indicated whenever there is unexplained central vision loss.

Figure 5  The Amsler’s chart used for testing the central vision of the eye. Figure from http://www.afv.org.hk/eyecare_frameset_e.htm

The patient is instructed to cover one eye at a time, and focus on the center dot to look for any potential signs of AMD like wavy, distorted or missing areas of vision. Nowadays this test is a standard for self-checking of visual function. In most practice, it is a rapid, concise but nonspecific method for detecting dysmorphopsia. However, the limitation is that it is a psychophysical test, which the result may be affected by the subjectivity of the patient. Apart from Amsler grid, indirect ophthalmoscopy is routinely used. It provides a stereoscopic overview of the posterior pole of the eye. This enables the ophthalmologist to look for clinical signs of AMD. Actually, it is a head-wearing device, which consists of an illumination system with a set of magnification lens for fundus examination. With such a viewing system, it enhances a better resolution for a global picture of the fundus. However,
the main limitation is a relative low degree of magnification, thus making it difficult to observe subtle changes in the macula. Besides, the resultant image obtained is reversed and inverted, which is rather inconvenient for interpretation. What’s more, pupil dilation is mostly required beforehand by mydriatic drops, which need the compliance of the patient during the procedure.

To get a fine resolution for the maculopathy in AMD, fundus photography is another tool used by ophthalmologist. It is a modern camera used to capture the image in the fundus. It can be recorded digitally and viewed on a computer monitor. Thus, the resultant image gives excellent resolution for any subtle changes in the macular area. However, the only concern for using this diagnostic tool is the expensive cost of equipment. Besides all the above techniques, fundus fluorescein angiography is the most useful diagnostic tool to confirm the diagnosis of AMD, especially in the presence of CNV (Dante et al, 1999). Practically, it is not only used to confirm the diagnosis of AMD but also serves as a guide to laser photocoagulation. Basically, it involves the intravenous injection of a fluorescent dye, which is immediately followed by serial fundus photography. This procedure is used to document retinal blood flows dynamics and to evaluate the integrity of the blood-retinal barriers. Thus, whenever a patient presents with clinical signs of exudation in the macula, a fluorescein angiogram should be performed to accurately locate CNV. However, the main pitfall is mainly related to its invasive nature. Occasionally, serious side effect such as brochospasm, anaphylaxis and cardiac arrest could be found (Richard, 1999). Thus, complete medical profile concerning about the patient should be obtained prior to FFA.
1.8 Treatment

Currently, there is no reliable therapy for dry AMD, and only about 5% of patients with wet AMD are candidates for appropriate treatment (Stokkermans, 2000a). Especially, it is only benefit those patients with a small area of CNV. In tradition, thermal laser photocoagulation with argon has been the standard of treatment. Unfortunately, this treatment is rather damaging and usually associated with an immediate drop of visual acuity. Apart from this drawback, most patients treated with thermal laser will have a recurrence of CNV within the first year after treatment. That is why there have been a number of clinical trials seeking for alternative treatments. On April 12, 2000, the Food and Drug Administration approved the use of Visudyne for photodynamic therapy (PDT) in neovascular AMD (Carl, 2000; Rivellese et al, 2000; Ursula et al, 2000; Shuler et al, 2001). Basically, photodynamic dye is infused intravenously and the choroidal neovascular membranes (CNVM) are treated with low intensity laser. The dye is thought to concentrate in the CNVM causing thrombus formation after laser activation, thus avoiding further leakages of CNV. With the development of PDT, it seems to be a milestone for AMD treatment. In Hong Kong, PDT for AMD is available in Prince of Wales Hospital recently. Apart from this revolutionary treatment, many new treatments are currently under investigation (Stokkermans, 2000b). These treatments include angiogenesis inhibitors, gene therapy, transpupillary thermotherapy, macular translocation surgery, prophylactic laser treatment of drusen and retinal epithelium transplants etc. Among all these areas, the potential use of anti-angiogenic gene therapy seems to be a promising therapy for retinopathy involving neovascularization. In AMD, increased vascular endothelial growth factor (VEGF) expression appears to be a major regulator for developing CNV, thus the control of VEGF may be the key to make photocoagulation more efficacious in inhibiting AMD-related CNV (Kim et al, 1999). With the use of retrovirus-mediated gene transfer system, it can transduce genes encoding anti-angiogenic factors in the
photocoagulation sites, thus further avoiding recurrence of CNV after treatment. Recently, studies of retinopathy have focused on using it in animal models. Conclusive findings were reported that gene transfer and stable expression were found selectively near site of CNV with relatively high efficiency (Murata et al, 1998). Though it is at a very early stage in the development of gene therapy for AMD, this lien of research may be very rewarding. With the introduction of PDT in AMD, it seems to make the dream of a 'cure' in AMD patient come true. However, extensive application of PDT has its own limitation because not every AMD patient is suitable for this treatment. Therefore, the most critical issue for dealing with this eye disease is a successful hunting of AMD gene in the future. This allows more advanced presymptomatic molecular testing to be done for screening those high-risk individuals of AMD. Thus, preventive measures can be taken for those identified individuals.

1.9 Diet and AMD

Cumulative evidence suggests that oxidative damage is an important factor in the pathogenesis of AMD (Beatty, et al, 2000). In the human body, there are two lines of defense against the damaging effect of reactive oxygen species. The first line of defense is antioxidant enzyme, such as superoxide dismutase, catalase and peroxidase, which decompose the reactive species. The second line of defense is antioxidant nutrients such as α-tocopherol, β-carotene, and ascorbate, which either inhibit the initiation of lipid peroxidation or break the propagation of oxidative chain reaction. Thus, the roles of these antioxidants in preventive eye care become a hot topic for research (Eye Disease Case-Control Study Group, 1993). As these nutrients are supplied through dietary intake, studies of association between these antioxidants and AMD are highly focused (Smith et al, 1997; Ho et al, 1991; Cohen et al, 1994). However, published reports are mostly limited to cross-sectional and case-control studies, and some previous studies failed to consider important potential confounders, thus
leading to inconsistent findings. In fact, some of these antioxidants (vitamin E, vitamin C, carotenoids, zinc) have been shown to have protective effects for AMD in certain studies (Age-Related Eye Disease Study Research Group, 2001). Even no one has proven conclusively such nutrients can prevent AMD, it is true that a healthy diet is important for us. At this time, there is insufficient evidence to recommend vitamin or mineral supplementation as preventive measures against AMD because effectiveness, appropriate dose and duration are still not established. Nonetheless, diet offers good potential for preventive strategies.

1.10 Lutein and zeaxanthin

Among different dietary antioxidants, carotenoids have shown a relatively consistent protective effect against AMD. Basically, carotenoids are lipophilic molecules, which can be classified as carotenes and xanthophylls (Deming et al, 1999). To a certain extent, they share a common structure containing a series of centrally located, conjugated double bonds (Figure 6). In fact, some carotenoids are open chain polyene molecules, while others have closed end groups. In term of polarity, carotenes such as β-carotene, α-carotene and lycopene are non-polar hydrocarbons. On the contrary, xanthophylls such as lutein, zeaxanthin, are rather polar because they contain hydroxyl group at their cyclic ring structure.

Unlike other provitamin A carotenoids, lutein and zeaxanthin cannot be converted to vitamin A. Their presence in tissues is due to dietary plant sources only, as they are not synthesized by animal tissues. Thus, plant foods become the primary dietary sources of carotenoids for most mammalian species. In nature, more than 600 carotenoids have been identified but only 50 are commonly found in fruits and vegetables consumed in our diets, and only 10 to 15 are absorbed sufficiently to be able to be detected in the serum. Generally, carotenoids are present in a wide variety of plant foods and may impart a yellow color to the plants. In particular, their concentration is high in leafy green vegetables such as spinach,
collards and kale. In a previous nutritional study, it was found that the highest amounts of lutein and zeaxanthin were found in egg yolk and in maize (Sommerburg et al, 1998). Besides, substantial amounts of these xanthophylls are also present in kiwi fruit, red seedless grapes, orange juice and zucchini. Thus, it is advisable to eat more plant foods and less meat in order to achieve a high intake of these.

For the study of maculopathy, much strenuous effort has been put on the role of lutein and zeaxanthin because they are the major carotenoids specifically concentrated in the macula. The cells of human macula selectively accumulate lutein and zeaxanthin (Handelman et al, 2000). By contrast, other carotenoids such as β-carotene and lycopene are virtually undetectable within the retina despite abundant dietary consumption and much higher blood levels. Thus, this specificity strongly implies that specialized binding proteins may be responsible for the uptake and stabilization of these macular carotenoids. Recent evidence suggested that substantial portion of the macular carotenoids is bound to structural protein called tubulin (Beatty et al, 1999). However, it is not clear yet by which mechanism these carotenoids accumulate in the ocular tissues from the immediate source of blood. Thus, it is worth to put more effort on this aspect to solve the underlying mechanism.
Figure 6  Structures of common carotenoids. Carotenes: (1) all-trans-β-carotene, (2) α-carotene, (3) all-trans-lycopene, (4) 9-cis-β-carotene; xanthophylls: (5) lutein, (6) zeaxanthin, (7) canthaxanthin, (8) β-cryptoxanthin. (Deming et al, 1999)
1.11 Action of carotenoids

For most carotenoids, their intense coloration is due to the extensive conjugation in their carbon skeleton. Even their biological functions are not fully understood, most of their characterized functions in biological systems are related to their conjugated double-bond structure. For example, lutein and zeaxanthin are major constituents of the yellow macular pigments in the human retina. These pigments play a protective role for the retina (Beatty et al, 1999). Basically, macular pigment is composed mainly of three isomeric carotenoids which are lutein, zeaxanthin and meso-zeaxanthin. They account for about 36%, 18% and 18% of total carotenoids in the retina, respectively (Landrum et al, 2001). Although macular pigment was spectroscopically detectable in all macular layers, dense pigmentation was found at the center of the fovea, especially in the photoreceptor axons of the foveola. In the past, many studies have concentrated on how and why these xanthophylls accumulate in the macula. In a monkey model, a previous study showed that the central peak of macular pigment extend about 100 μm from the foveal center (Landrum et al, 1999b). Besides, other study reported an average mass of carotenoids per unit of retinal area of 1.33 ng/mm² at the foveal center compared with 0.81 ng/mm² at an eccentricity of 1.6-2.5 mm. This showed that their concentrations diminish with distance from the centre of the macula. Although lutein and zeaxanthin both reach their maximum concentration at the foveola, they demonstrate specific pattern in their distribution. In general, zeaxanthin is the dominant carotenoid at the foveola (Krinsky et al, 2002). With increasing eccentricity, zeaxanthin declines more rapidly than lutein, which leads to lutein being the dominant carotenoid in the perifoveal zone. Even though the distribution of lutein and zeaxanthin in the macula have been described, but the function of macular pigment still remains uncertain. It was proposed that these pigments could act as a broad band optical filter. It is well suited as a filter of incoming blue light for several reasons. Firstly, the absorbance spectrum of macular pigment peaks at 460 nm (Figure
7). This can reduce the sensitivity of the macular region to short wavelength light, which is the most damaging spectrum for the retina at wavelengths between 350 nm and 450 nm (Ham et al., 1982; Ham et al., 1976). Several studies show clear evidence that macular pigment attenuates photic damage in the human retina. Scattering and chromatic aberration of blue light may be minimized by these macular pigments.

Besides, many studies have shown that these retinal carotenoids are potent antioxidants (Edge et al., 1997; Landrum et al., 1999a). In the retina, generation of reactive oxygen species can occur as the byproducts of cellular metabolism or the result of photochemical reactions. For these reactive species, they are partially reduced oxygen species containing one or more unpaired electrons, such as superoxide anion, hydroxyl radical.

Figure 7 Absorption spectrum of macular pigment as plotted by Wyszecki and Stiles (line) and Werner et al (points). (Beatty et al., 1999)
Other reactive species with their full complement of electrons in an unstable or reactive state are also found in the body, such as singlet oxygen, hydrogen peroxide. All these molecules are highly reactive and can be generated by chain reactions. They can cause detrimental damage because they readily react with lipid and protein, causing impaired cell function or cell death in our body. Especially, the retina is susceptible to damage by these reactive species because it contains high levels of polyunsaturated fatty acids, which are easily oxidized by these damaging radical species. To explain the underlined antioxidant properties of carotenoids, it was proposed that lutein and zeaxanthin could interfere with the chain reaction by either reducing the rate of chain propagation or by participating in a chain terminating chemical event (Landrum et al, 2001). They can also react directly with peroxy radicals producing a highly resonance-stabilized carotenoid radical in which the unpaired electron is delocalized over the conjugated polyene. This enhances the stability of the radical, thus providing opportunity to allow other naturally occurring reductants (tocopherol and ascorbic acid) to react with the radical within the tissues. It seems that such explanation is reasonable and support the hypothesis that retinal carotenoids can act as free radical scavenger. With these diverse protective roles, lutein and zeaxanthin were strongly believed that they have important role in prevention of AMD. Therefore, it has been suggested that eating green leafy vegetables, which are rich in lutein and zeaxanthin, may decrease the risk for age-related macular degeneration. In a previous study, the Case Control Study Group for Eye Diseases showed that a high dietary intake of carotenoids, was associated with 43% lower risk for AMD. That is the reason why dietary modification or supplementation with lutein and zeaxanthin become popular and highly promote in health food product market.
1.12 Bioavailability of macular carotenoids

To increase our understanding of the potential benefits of carotenoids, it is important to obtain more insight into their bioavailability from foods and the factors that determine the bioavailability (Moeller et al, 2000). In Figure 8, pathway of carotenoid absorption and metabolism is shown for illustration.

![Diagram of carotenoid absorption and metabolism]

Figure 8 Pathway of carotenoid absorption and metabolism: C, carotene; X, xanthophylls; LPL, lipoprotein lipase; HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein. (Deming et al, 1999)

In the human body, the absorption pathway of carotenoids is similar to the route of dietary fats because they are lipid-soluble. For optimal absorption of carotenoids, disruption of the food matrix and release of carotenoids are critical steps to be considered (Zaripheh et al, 2002). Carotenoids are partially released from the food matrix by mastication, gastric action and digestive enzymes in the early digestive process. However, various factors can
affect the bioavailability of carotenoids. For example, the amount of carotenoids released from the food matrix is quite variable and depends on whether they are complexed with other components, such as proteins, dietary fibre etc (Castenmiller et al, 1999). It is because these will hinder the release of carotenoids from such complex. Another important issue is whether the carotenoids are present in the crystallized form, dissolved in dietary oils or if the foods are finely chopped and cooked (Stahl et al, 1992). Hence, the mode of treatment during food preparation becomes an important aspect for carotenoid bioavailability. For instance, mechanical homogenization, heat treatment, and addition of fat during the processing of vegetables are feasible techniques to enhance carotenoids bioavailability.

After the release of carotenoids from the food matrix, it is solubilized into lipid globules of varying sizes in the stomach. Solubilization of these molecules into lipid emulsions is believed to be dependent on the specific polarity of each individual molecule. For instance, those non-polar carotenoids will migrate to the triacylglycerol-rich core of the particle, while the more polar xanthophylls orient at the surface monolayer along with proteins, phospholipids etc. After solubilization into lipid emulsions, these particles are transported from the stomach to the duodenum of the small intestine. In the duodenum, the presence of dietary fat triggers the release of bile acids from the gall bladder and regulates levels of pancreatic lipase. The function of bile acids is to reduce the size of lipid particle and stabilize it into mixed micelles. In the presence of pancreatic lipase, it can hydrolyze dietary triacylglycerols into free fatty acids and monoglycerides. Thus, carotenoids are solubilized into mixed micelles along with dietary triacylglycerols, and their hydrolysis products. It was suggested that optimal absorption of carotenoids may require an intake of as little as 5 g of fat per meal. Hence, ingestion of fat along with carotenoids is thought to be crucial for effective absorption. Besides, the type of fat present in the diet also influences its bioavailability. That means for those unabsorbable, fat-soluble compounds, they can reduce their absorption and
result in a reduced carotenoid bioavailability.

Absorption of carotenoids into the intestinal mucosa follows a similar pathway to other lipid components within the mixed micelle. It is thought that absorption simply mediated by passive diffusion due to the concentration gradient between the micelle and the cell membrane of the enterocyte (Berg et al, 2000). Its uptake into the enterocyte will not be entirely metabolized or absorbed into the body. It is because some may be lost in the lumen of gastrointestinal tract due to normal physiological turnover of the mucosal cells. Having completed the uptake of carotenoids into the enterocyte, β-carotene-15,15'-dioxygenase can cleave some of the absorbed β-carotene and other provitamin A carotenoids to vitamin A. On the other hand, some carotenoids and other lipid molecules are assembled into chylomicrons in the Golgi apparatus of the enterocyte and released into the lymphatics (Deming et al, 1999). In general, there is preferential uptake of polar xanthophylls from the intestinal lumen into chylomicrons, even in the presence of high amount of non-polar carotene. However, the exact mechanism of carotenoid translocation within the enterocyte is not known. Later on, chylomicrons are secreted from the enterocyte and transported to the liver via lymphatic circulation. Prior to hepatic uptake, chylomicrons in the blood are rapidly degraded by lipoprotein lipase that is associated with tissue endothelium, and are transformed into chylomicron remnants. During this process, some carotenoids may be taken up by extrahepatic tissues. However, most chylomicron remnants deliver carotenoids to the liver where they are stored or resecreted into the bloodstream in very low density lipoproteins (VLDL). Subsequently, circulating VLDL are delipidated to low density lipoprotein (LDL). Carotenoids released from lipoproteins, especially LDL, are taken up by extrahepatic tissues. In humans, dietary carotenoids accumulate in many tissues including the liver, adipose, serum, breast milk, adrenal, prostate, macula, kidney etc. It was suggested that certain carotenoids may exert a biological effect in one tissue over another. However, factors
controlling tissue uptake, recycling back to the liver and excretion are not fully understood. That is why most current studies want to determine whether particular carotenoids are simply present and serve as biomarkers of plant foods intake or function as specific modulators of disease in the tissues.

For a normal Western diet, it contains 1.3-3 mg/day of lutein and zeaxanthin combined. It was estimated that the ratio of lutein to zeaxanthin in the diet ranges from about 7:1 to 4:1 (Johnson et al, 2000). However, not much dietary intake study has been done for the Chinese in Hong Kong. Nonetheless, these studies seem to be very informative. In fact, levels of intake vary considerably across individuals and population subgroups. In human serum, lutein and zeaxanthin are major serum carotenoids, along with beta carotene, alpha carotene and lycopene. In previous study it was found that serum lutein dominates over zeaxanthin in the human serum (Table 1) (Age-Related Eye Disease Study Research Group, 2000). The ratio of serum lutein to zeaxanthin is somewhat variable, ranging from 2.7 to 4.5:1, which depends upon diet and lifestyle. Recently, a study showed that the normal serum levels of lutein were found to range from 1.02 to 4.47 X 10^{-4} mmol/L, with an average value of 2.46 X 10^{-4} mmol/L (Bone et al, 2000). In the same subjects, they were found to have zeaxanthin concentrations that ranged from 0.546 to 1.76 X 10^{-4} mmol/L and averaged 8.98 x 10^{-5} mmol/L. Dietary intake of foods rich in these carotenoids has been shown to influence serum concentrations in a positive manner. However, individual variation in serum response to increased intake has been observed and may be due to factors such as varying rates of absorption and tissue uptake. Besides, in some research findings, it was found that serum levels associated with normal diet are far below the maximal levels achieved with lutein or zeaxanthin supplementation. However, whether the increased level of these xanthophylls in the serum will finally benefit for the prevention of AMD is still doubtful. Therefore, much clinical trial should be done in this aspect.
<table>
<thead>
<tr>
<th>Normal Diet</th>
<th>Ratio of lutein to zeaxanthin</th>
<th>Levels of lutein (mmol/L)</th>
<th>Levels of zeaxanthin (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>7:1 to 4:1</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>2.7 to 4.5:1</td>
<td>1.02 to 4.47 X 10^{-4}</td>
<td>0.546 to 1.76 X 10^{-4}</td>
</tr>
</tbody>
</table>

Table 1  Summary showing the serum level of lutein and zeaxanthin (mmol/L) and ratio of lutein to zeaxanthin in normal diet and serum. NR: Not reported.

1.13 Wolfberry

In pharmaceutical Latin, wolfberry is named as *Fructus Lycii*. Most of the Chinese refer to it as Gou Qi Zi (Kei Tze'). This delicious fruit is very widely used as an excellent blood tonic (Herbasi Chinese herb database). Regular consumption of Kei Tze is traditionally believed to lead to a long, vigorous and happy life. Particularly, it is also said to brighten the eyes and improve vision. However, in the past, not much scientific clinical trial has been conducted to demonstrate its beneficial effect on visual health. Besides, this fruit is widely believed to increase sexual fluids and enhance fertility. Apart from these, it is commonly used by first trimester mothers to prevent morning sickness. Thus, it has long been a favorite 'herb' for the Chinese. However, there is lack of strong evidence to prove its effectiveness in a scientific way. Especially, the underlying mechanism and the active ingredient(s) for its reported functions are not known. To put in a critical manner, it seems that there is lack of quantitative and qualitative measurement of Chinese herb, which hinders the development of Traditional Chinese Medicine compared with the Western Medicine. It is believed that more scientific analysis of *Fructus Lycii* enables us to demonstrate its diverse
therapeutic effectiveness in more intervention studies.

1.14 Source of wolfberry

Because of its great fame over many centuries, wolfberry has been collected and cultivated in almost every region of China. In most areas of China, it grows on hillsides and ridges. Generally, the best Kei Tze grows in cool climatic areas. It is harvested in the summer and autumn when the fruit is mature. After harvesting, it is spread thin on bamboo mats and dried in semi-shady sunlight. Basically, there are two main types of *Fructus Lycii*. The first is called NingXia Kei Tze, which is the preferred herb of herbal connoisseurs. It is characterized by big appearance with thick fruit meat and few seeds (Figure 9). For the larger ones, they tend to be sweeter, and have a juicier texture. In the selling market, NingXia Kei Tze is further graded according to size. For example, grade 1 NingXia Kei Tze has no more than 370 grains (berries) per 50 grams. Grade 2 has no more than 580 grains per 50 grams. Grade 4 has no more than 1100 grains per 50 grams and no more than 15% damaged fruits (Quality Chinese Herbs – Lycium Fruit). The second type of *Fructus Lycii* is known as Blood Lycium. Comparing with NingXia Kei Tze, their qualities are rather inferior. They tend to be relatively small and have numerous seeds. Moreover, they have tougher skin and are considerably less sweet compared with NingXia Kei Tze. Most often, they have distinct tartness that overwhelms the sweetness. Owing to the lower cost of Blood Lycium, most producers prefer to select it for packaged products.
1.15 Constituents of wolfberry

Focusing on the constituents of Kei Tze, it has the highest content of beta-carotene among all foods on earth. Thus, it is believed to be a powerful antioxidant that has a significant protective role in our body. Besides, vitamin B1 and B2 content is significantly high. The fruit also contains vitamin C, beta-sitosterol (an anti-inflammatory agent), linoleic acid (a fatty acid), zeaxanthin and betaine (0.1%). Not only the above constituents but also it contains 18 kinds of amino acids. Among these amino acids, there are 8 amino acids which are indispensable for the human body, such as isoleucine and tryptophan. Besides, 50% of these amino acids are free amino acid, which are readily used by the human body. In addition, it contains numerous trace elements such as zinc, iron and copper. For example, mature Kei Tze contain about 11 mg of iron per 100 grams of fruit. Therefore, it is believed that Fructus Lycii is nutritious enough as a blood tonic which contain significant amount of antioxidant, amino acid and mineral etc.
1.16 Evidence of benefit for ocular health

Chinese people have been using wolfberry to prevent vision degeneration or treat mild blurry vision for more than one thousand years. It is commonly used in home cooking because of its flavour as well as general health benefit. Besides, it is also used in herbal formulas to improve visual acuity. However, the rationale for its reported benefit to eyes remained a mystery until modern science discovered that wolfberry fruit contained substantial amounts of pro-vitamin A such as beta-carotene and cryptoxanthin. They can be transformed into vitamin A under the influence of human liver enzymes. Therefore, vitamin A ultimately plays a major influence in Lycium's actions. However, these are not the only constituents for the beneficial evidence of Fructus Lycii in ocular health. As mentioned before, it was found that lutein and zeaxanthin were the major carotenoids concentrated in the macula. This specific accumulation of these two carotenoids in the macula has led to hopes that dietary supplementation with these carotenoids may increase the macular pigment density or reduce the risk for AMD. In the past, the plasma carotenoid and macular pigment density were measured in a supplementation study of two subjects receiving 30 mg lutein (as a marigold lutein ester extract suspended in canola oil) daily for 140 days (Landrum et al, 1997). It was found that serum lutein levels rapidly increased 10-fold from 0.2 - 0.3 μmol/L in the first week and maintained that level for the remainder in the study. Macular pigment density, as estimated by heterochromatic flicker photometry, showed a slower response than the serum, starting to increase after approximately 20 days. Hence, it is believed that dietary supplementation with lutein and zeaxanthin are beneficial to ocular health as it may increase the macular pigment density after certain period of supplementation.

In our diet, various vegetables and fruits are good sources of lutein and zeaxanthin. Many studies have been done to measure the amount of different carotenoids in selected plants by HPLC. Compared with other vegetables and fruit in Table 2, the content of
zeaxanthin in *Fructus Lycii* was much higher (up to 5 mg/100g) (Wolfgang et al). On the contrary, it has a very low content of lutein, which is less than 1%. Besides, it was found that the zeaxanthin exists in ester form in *Fructus Lycii*. This natural occurrence of the ester form accounts for the stability of medicinal effect of *Fructus Lycii* in the dried berries. It is because the hydroxyl groups in zeaxanthin can be damaged by oxidative degradation. Therefore, acylation of zeaxanthin should prevent oxidation of the hydroxyl group. In nutrition aspects, it seems to be a fascinating natural design to provide one stable source of zeaxanthin for ocular health. However, not much clinical trial has been done for the effect of macular pigment density and serum xanthophylls after supplementation with *Fructus Lycii* in human body.

<table>
<thead>
<tr>
<th>Vegetables</th>
<th>Lutein</th>
<th>Zeaxanthin</th>
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<th>α-carotene</th>
<th>Cryptoxanthin</th>
<th>Lycopene</th>
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<td>3.3</td>
<td>np</td>
<td>np</td>
<td>np</td>
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<td>np</td>
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<td>3.9</td>
<td>nd</td>
<td>np</td>
</tr>
<tr>
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<td>0.4</td>
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<td>0.06</td>
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<tr>
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<td>1.6</td>
<td>0.2</td>
<td>0.2</td>
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<tr>
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<td>np</td>
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<td>np</td>
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<tr>
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</tr>
</tbody>
</table>

**Abberivations:** nd = not done; np = not present; nr = not reported.

Table 2 Amount of carotenoids (mg/100g) in selected plants. (Wolfgang et al)
Recently, serum and retinal levels of zeaxanthin and lutein were studied after feeding rhesus monkeys an extract of *Fructus Lycii* for 6 weeks (Leung et al, 2001). It was found that serum levels of zeaxanthin and lutein in the feeding group were significantly higher than those of the control group. In particular, the serum zeaxanthin concentration after feeding treatment for 6 weeks increased by 2.5 times compared with the basal level. However, a significant increase in serum lutein was also noted after supplementation with *Fructus Lycii*. It seems a contradictory finding because the content of lutein is low (less than 1%) in *Fructus Lycii* and it is an unlikely source to explain for the increased serum lutein in this study. Thus, it was suggested that zeaxanthin can be converted to lutein, but there is no evidence in the literature to suggest zeaxanthin is converted to lutein in serum or retina, whereas the reverse has been proposed. In fact, lutein and other carotenoids are present in the daily fruits in the monkey diet. It may be act as a source for the increased of serum lutein in the study. Other possible explanation has been suggested that zeaxanthin or other components in the extract of *Fructus Lycii* may enhance the absorption of lutein. It is also possible that there is a metabolite of zeaxanthin coeluting with lutein that cannot be separated by HPLC. However, there is no confirmed explanation for the increased serum lutein after supplementation in this study. In the HPLC measurement, it showed that the zeaxanthin level in the maculae was elevated by feeding supplement rich in zeaxanthin, *Fructus Lycii*. The average zeaxanthin content in the maculae of the treatment group was 2 times higher than those in the vehicle-treated (control) group. The elevated level of zeaxanthin after *Fructus Lycii* supplementation indicated preferential uptake of zeaxanthin in the central macula. The absence of any carotenoids in the peripheral or equatorial regions further suggests specific uptake mechanisms exist only at the center of the retina. It has to be noted, however, that only this single animal study of absorption of carotenoids from Lycium has been performed to date.

With the great fame of *Fructus Lycii*, it seems to be an important ingredient in
Chinese herbal medicine for various ocular diseases such as cataract, AMD, glaucoma and retinitis pigmentosa. Whether *Fructus Lycii* is beneficial in the treatment of these ocular diseases remains to be studied. However, it is believed that the abundant zeaxanthin in this berry and the ready absorption of its zeaxanthin into serum and the macula of primates may be beneficial in protecting the retina against free radical and blue light damage. In conclusion, much more in the way of clinical supplementation trials, particularly in human studies, should be done to investigate the potentially beneficial effects of *Fructus Lycii* for ocular health.
1.17 Aims and significance of the study

The aims of this study are to investigate in vitro total antioxidant power of *Fructus Lycii* products and to investigate the effect of supplementation with *Fructus Lycii* on blood antioxidant status, including lutein and zeaxanthin. This study has significance because of the potential for zeaxanthin-rich Kei Tze to maintain the macular pigment, protect the RPE and lower risk of AMD, and because, to date, there are no human data on the effect of supplementation with Kei Tze on plasma lutein and zeaxanthin or antioxidant levels. If it can be demonstrated that supplementation with *Fructus Lycii* causes an acute or cumulative increase in plasma carotenoid and/or antioxidant status, then a good case could be made for a supplementation study investigating its longer-term effect on macular pigment density and visual acuity in subjects at high risk of AMD.

There are 2 objectives in this study:

1. To investigate in vitro studies of total antioxidant power of different brands of Kei Tze products,
2. To investigate the effect of supplementation with Kei Tze on blood antioxidant status, including lutein and zeaxanthin.

The study will be divided in 3 parts

1. In vitro studies of total antioxidant power of different brands of Kei Tze products,
2. Set up and evaluation of HPLC method to measure plasma lutein and zeaxanthin,
3. 28 days in vivo supplementation of Kei Tze with a single-blinded, placebo-controlled human intervention of parallel design.
2. Materials and methods

2.1 In vitro studies of total antioxidant power of Kei Tze products

2.1.1 Preparation of Kei Tze extracts

4 different brands of Kei Tze products, a) Quality Fructus Lycii (Yue Hwa Chinese Product Ltd, HK), b) Natural Wolfberry (Organic Garden Ltd, HK), c) Rich Nature™ Wolfberry (Rich Nature Labs Inc. USA) and d) NingXia Kei Tze (from local herb store) were purchased from local market. These 4 different brands of Kei Tze were tested for the in-vitro total antioxidant capacity. Results presented were the total antioxidant content as the FRAP value.

10% w/v extract was prepared by homogenizing 10 g of Kei Tze in 100 ml extraction medium using electric blender for 2 minutes. Kei Tze extracts were prepared in 4 types of medium as follows: boiling distilled water, distilled water at room temperature, 300 mM acetate buffer pH 3.6 at room temperature and 100% ethanol at room temperature. Homogenates were filtered, and the total antioxidant capacity was measured in triplicate immediately afterwards using FRAP assay (Benzie & Strain, 1999b).

2.1.2 Ferric Reducing/Antioxidant Power (FRAP) Assay

All reagents and solutions were prepared in Milli-Q water. Milli-Q water was made from a Millipore ultra-pure water system (Millipore Corp., USA). FRAP reagents were as follows: 300 mmol/L acetate buffer, pH 3.6, prepared by dissolving 3.1 g sodium acetate trihydrate in distilled water, with 16 ml glacial acetic acid (BDH Laboratory Supplies, Poole, UK) added and made up to 1 litre with distilled water; 10 mM-2,4,6 tripyridyl-s-triazine (Fluka Chemical, Buchs, Switzerland) solution in 40 mM-HCL. 20 mM-FeCl₃.6H₂O (BDH Laboratory Supplies) solution in distilled water. Working FRAP reagent was prepared as needed by mixing 20 ml acetate buffer with 2 ml 2,4,6 tripyridyl-s-triazine solution and 2 ml
For FRAP analysis, 100 µl of each freshly prepared Kei Tze extract was loaded on Cobas Fara centrifugal analyzer (Roche Diagnostics Ltd, Basel, Switzerland). The assay was performed as described in detail elsewhere (Benzie & Strain, 1997). In brief, antioxidants in the sample reduce a ferric-tripryridyltriazine complex, present in stoichiometric excess, to the blue coloured ferrous form. The change of absorbance at 593 nm over 4 minutes was proportional to the total FRAP value of the antioxidants in the sample. The FRAP value was obtained by comparison of absorbance to change to that induced by a solution of Fe²⁺ ions. The FRAP value in µmol/L was calculated by simple comparison of 0-4 minutes change in absorbance at 593 nm of the test sample and that of a Fe²⁺ calibrator, as follows:

\[
\frac{0-4 \text{ min } \Delta A_{593 \text{ nm}} \text{ of the test sample} \times \text{ FRAP value of standard (µM)}}{0-4 \text{ min } \Delta A_{593 \text{ nm}} \text{ of standard}}
\]
2.2 Set up and evaluation of HPLC method to measure lutein and zeaxanthin

2.2.1 Measurement of plasma lutein and zeaxanthin

Chemicals

Lutein and zeaxanthin were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ, USA), absolute ethanol, tetrahydrofuran (THF) and hexane (all Chromasolve for HPLC) were from Riedel-de Haen (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), butylated hydroxytoluene (BHT) was from Sigma (St. Louis, MO, USA), ascorbic acid was from E. Merck (Darmstadt, Germany) and acetonitrile (HiperSolv for HPLC) was from BDH Laboratory Supplies (Poole, England). Milli-Q water was made from a Millipore ultra-pure water system (Millipore Corp., USA).

HPLC conditions

Chromatography system included Alliance 2695 Separations Module with temperature-controlled column chamber and autosampler (Waters, Milford, MA, USA), Waters 2996 Photodiode Array Detector and Waters Empower Pro PDA software (version 5.00.00.00), a reversed-phase Luna C$_{18}$ analytical column (5 µm, 250 x 4.6 mm i.d.; Phenomenex, Torrance, CA, USA) and a cartridge guard column (Security Guard, C$_{18}$, cartridge 4 x 3.0 mm i.d., from Phenomenex). The mobile phase was 0.1% w/v BHT in acetonitrile, which was filtered and degassed through a Millipore membrane (type GV, pore size 0.22 µm, Millipore Corp., USA) before use. The detection was set at 450 nm. The flow rate was time-programmed at two speeds, 1.0 mL/min for 0 - 12 minutes and 2.5 mL/min for 13 - 60 minutes. The columns worked under room temperature but the cooling chamber of the autosampler was set at 4°C.
Preparation of stock and working standard solutions

All preparations involving lutein and zeaxanthin were sheltered from strong light. Stock solutions (200 μg/mL or 351.6 μmol/L) of lutein and zeaxanthin were first prepared in 0.1% w/v THF. With dilutions (in 0.1% w/v BHT in ethanol) of these stock solutions, a working solution containing lutein (0.5 μg/mL or 0.88 μmol/L) and zeaxanthin (0.25 μg/mL or 0.44 μmol/L) was made up in 0.1% w/v BHT/ethanol. Both the stock and the working solutions were stored at -20°C.

Preparation of calibrators, pooled plasma and controls

From the working solution of lutein (0.5 μg/mL or 0.88 μmol/L) and zeaxanthin (0.25 μg/mL or 0.44 μmol/L), concentrations of lutein (0 - 0.5 μg/mL or 0 - 0.88 μmol/L) and zeaxanthin (0 - 0.25 μg/mL or 0 - 0.44 μmol/L) were diluted freshly with 0.1% w/v BHT/ethanol. By spiking into pooled human plasma, these solutions, referred to as calibrators, were used to construct the calibration curve. The calibrators were protected from strong light during preparation.

The pooled plasma for calibration was obtained from healthy human subjects. Venous blood from the antecubital vein of the subjects was collected into commercially-available heparinised tubes, chilled in ice-water and kept in the dark until plasma was separated from cells. Separation of plasma from blood was performed by centrifugation at 2500 g, 4°C, for 10 minutes; the separated plasma was then aliquoted and stored at -70°C until assayed with no intermediate thawing and refreezing. Similarly, heparinised human plasma samples were collected, aliquoted and stored at -70°C.

Low and high plasma controls were prepared by spiking known amount of lutein and zeaxanthin into pooled plasma. They were aliquoted and stored with samples at -70°C.
Procedures for calibration, plasma samples and plasma controls

The extraction procedures were modified from those performed by Aebischer et al., 1999. 200 μL of pooled plasma (in a 1.5 x 10 cm glass, screw-capped test-tube) was first mixed with 200 μL of Milli-Q water containing ascorbic acid (0.1 % w/v). To this solution, 20 μL of one of the calibrators and 380 μL of 0.1% w/v BHT/ethanol were added. The mixture was vortexed for 30 seconds following by addition of 800 μL of hexane containing BHT (0.1 % w/v) and rolled for 30 minutes on a horizontal mixer, Denley Spiramix 5 (Denley Instruments Ltd., England). The suspension was then centrifuged in a Jouan CR4-12 centrifuge (Jouan Ltd., Herts, France) at 2000 rev./min, 4°C, for 10 minutes. A 400-μL aliquot of hexane (upper layer) containing the extracted lutein and zeaxanthin was withdrawn and evaporated to dryness at ~40°C under a slow stream of nitrogen. The dry residue was temporarily stored in an ice-water bath and protected from light. The residue was vortexed first with 20 μL of THF (containing 0.1% w/v BHT), following by addition of 80 μL of mobile phase. The mixture was again well vortexed in order to re-dissolve the residue. These extraction procedures were repeated for the remaining calibrators. For the sample or control extraction, 200 μL of plasma sample or control was mixed with 200 μL of 0.1% w/v ascorbic acid/water and 400 μL of 0.1% w/v BHT/ethanol in the first step. Finally, 25 μL of each extracted calibrator, sample or control was injected into the HPLC system. Peak areas of lutein and zeaxanthin were recorded from each chromatogram. By plotting the peak area of lutein or zeaxanthin against concentration of calibrator spiked (i.e., concentration of lutein or zeaxanthin spiked into the pooled plasma), calibration curve of lutein or zeaxanthin could be constructed. The concentration (μg/mL or μmol/L) of lutein or zeaxanthin in each sample was calculated by dividing the peak area of the lutein or zeaxanthin in the sample with the slope of the lutein or zeaxanthin curve respectively.
Linearity of calibration, within-day and between-day precision of assay

The linear range was determined by direct injections of standards at various concentrations. Linearity of response was assessed from the coefficient of correlation (r) of the regression line. Six replicates of each calibrator at various concentrations were used to assess the within-day precision. The slopes from six consecutive day assays were used to assess the between day precision.

Detection limits of lutein and zeaxanthin

Stock solutions (200 μg/mL or 351.6 μmol/L) of lutein and zeaxanthin were 10-fold serial diluted (decreasing concentrations). The diluted standards were injected in sequence of decreasing concentrations to measure the detection limits. The signal to noise ratio was at least 2:1 for the limits.

Recoveries of lutein and zeaxanthin in plasma

Pooled plasma was spiked with lutein and zeaxanthin standards to make two elevated levels of lutein (100 and 300 ng/mL or 0.18 and 0.53 μmol/L) and zeaxanthin (25 and 75 ng/mL or 0.04 and 0.08 μmol/L). The neat (n = 4) and spiked plasma (n = 4) were then assayed. By comparing with direct injections of pure standards (n = 4 each), recoveries were assessed.

2.2.2 Hydrolysis of Kei Tze extract

To determine the lutein and zeaxanthin content of Rich Nature™ Wolfberry used in our supplementation study, 2 grains of Kei Tze (dry weight 0.405g) was cut and blended in mortar. It was then homogenized in 1 ml 6N sodium hydroxide (NaOH) and 1 ml of absolute ethanol containing 0.1% BHT. The mixture was subjected to ultrasonic for 3 minutes and
followed by incubation at 50°C for 3 hours. At the end of the incubation time, 1 ml of 6N hydrochloric acid (HCL) was added to the mixture for neutralization. The final pH of the mixture was adjusted with pH paper to 7.

2.2.3 Measurement of lutein and zeaxanthin content in Kei Tze

After neutralization, 2 ml hexane containing 0.1% BHT were added to the mixture for extraction of carotenoid. The sample was mixed by Denley Spiramix 5 (Denley Instruments Ltd., England) for 30 minutes and all samples were protected from light by covering with aluminium foil. After 30 minutes, the suspension was then centrifuged in a Jouan CR4-12 centrifuge (Jouan Ltd., Herts, France) at 2000 rev./min, 4°C, for 10 minutes. 1 ml of the hexane layer were removed and evaporated to dryness under a stream of nitrogen at 40°C using N-EVAP from The Meyer Associates Inc. (South Berlin). The residue was protected from light and kept in iced water. It was followed by re-dissolved in 20 μl of THF and followed by adding 80 μl of 100% acetonitrile containing 0.1% BHT. The isocratic mobile phase for chromatography was 100% acetonitrile containing 0.1% BHT. Each calibrator/sample extract was transferred to a small glass vial fitted with a micro glass insert and the vial was placed inside the HPLC autosampler at 4°C. 5 μl of each calibrator/sample was injected into the HPLC system. The content of lutein and zeaxanthin in Kei Tze (μg/g of Kei Tze) can be calculated by using the calibration curves. Extracted samples were stable for at least 8 hours at room temperature when protected from light.
2.3 28 days in vivo supplementation of Kei Tze with a single-blinded, placebo-controlled human intervention of parallel design

2.3.1 Subjects

The study design was a single blinded human intervention trial of parallel design for 28 days duration. A total of 27 apparently healthy volunteers were recruited with their informed consent. The age range of the subjects was 18 to 48 years (mean (SD) 27.6(9.46)) and BMI of 17.9 to 24.7 kg/m² (mean (SD) 20.88(2.2)). They were fully explained the details of the study with information sheet (Appendix I). Participants were required to complete questionnaire (Appendix II) providing information whether they met the selection criteria for this in vivo study. Selection criteria included that they should be non-smoker, free of history of glaucoma, cataract, age-related macular degeneration or other retinal diseases. Exclusion criteria included taking regular vitamin C and vitamin E capsule and carotenoid-rich supplement. For example, carrot, maize, Kei Tze etc. Subjects were allocated on a non-selective basis, to be in either a control group n = 13 (no Kei Tze supplement) or the study group n = 14 (15 g/day of dried Kei Tze berries).

2.3.2 Supplementation method

After in vitro-studies of Kei Tze, Kei Tze with the highest FRAP value was chosen as the supplement. Each volunteer in the study group was provided with 14 days supply (210g) of Kei Tze berries and instructions on how to prepare their daily supplement (Appendix III, Chinese version). After 14 days, they needed to return the empty bottles and the remaining 14 days (210g) Kei Tze were given to them. In brief, two spoons (spoon of standard size was supplied) of berries were added to one cup (around 200 - 300 ml) of boiling water and left to cool for around 15 minutes. The softened berries were then mashed with the back of spoon, and the berry and the ‘tea’ were ingested. In the study group, volunteers were requested to
take the Kei Tze in the evening, shortly after their evening meal, every day for 28 days. This time was chosen, and the berries fragmented, because there were reports that indicate carotenoids are more bioavailable in fragmented foods and if taken with fat. Berries were put into boiling water because, on the basis of the in vitro study, the antioxidant content of the tea was optimized. For the placebo group, no Kei Tze was taken after each dinner, but only a cup of warm water.

Fasting blood (around 5 ml each, taken around 9am, after an overnight fast and before breakfast) was taken for each individual at baseline and after 28 days. Blood was collected into commercial heparinised blood collection tubes. Urine and saliva were also collected in commercial collection plastic container. Follow-up of each participant was done weekly (at day 7, day 14 and day 21) during the 4 weeks of supplementation by means of telephone call or e-mail to check compliance and general health status of volunteers. They were asked whether they have any symptoms such as chills, fever, sore throat, vomiting, diarrhoea and skin rash etc, after they started their supplementation. Immediate follow up would be done once the participant complained for any adverse effect after starting the supplementation.

2.3.3 Sample Analysis

Blood samples were kept at 4 °C until seperation, which was within 3 hours of collection. Plasma was used immediately thereafter for the measurement of ascorbic acid concentration and the total antioxidant capacity (as the FRAP value). Washed erythrocytes and aliquots of plasma were stored at -70°C for other biochemical variables, all of which were measured within 3 months. Urine was tested on the day of collection for FRAP value, creatinine and H₂O₂ level. Saliva was also tested on the day of collection for FRAP value.

To measure plasma antioxidant capacity, and to assess absorption and bioavailability of Kei Tze antioxidants, FRAP assay was used. If dietary antioxidants were absorbed and
enter the systemic circulation, there would be an increase in plasma antioxidant capacity, the magnitude of increase reflecting the amount of antioxidant absorbed. If these antioxidants were also excreted, there would be an increase in urinary antioxidant capacity. The FRAP assay enables objective and highly reproducible results to be obtained on complex biological samples, it is simple and rapid enough to be performed on freshly collected specimens. In this study, the Ferric Reducing/ Ascorbic acid (FRASC) assay was also used to measure both the total antioxidant capacity (as the FRAP value) and the ascorbic acid concentration simultaneously (Benzie & Strain, 1997). FRASC was performed as described previously in detail (Benzie & Strain, 1999b).

Several biomarkers of oxidant: antioxidant balance were measured pre- and post- 28 days supplementation. These included: plasma alpha tocopherol (vitamin E; total and lipid standardized), lutein, zeaxanthin (total and lipid standardized) and uric acid, as well as red blood cell SOD and whole blood GPx. Total cholesterol and triglycerides were measured to lipid standardize alpha tocopherol. SOD and GPx were measured using commercial kit methods (Randox, Co Antrim, Northern Ireland). Haemoglobin (Hb) was measured, and SOD results were expressed as IU enzyme activity/g Hb. GPx results were expressed as IU enzyme activity/L of whole blood. All these biomarkers were measured to assess blood antioxidant status in pre- and post-28 day’s supplementation blood samples. Plasma alpha tocopherol, lutein and zeaxanthin were measured by HPLC according to the following in-house standard protocols. Plasma uric acid was measured using a commercial kit method (Roche, Basel, Switzerland). Plasma total cholesterol (TC) and triacylglycerol (Tg) were measured using commercial test kit methods (Roche, Basel, Switzerland) and used for lipid standardization of plasma alpha tocopherol concentrations. Urine creatinine was measured by commercial test kit (Roche, Basel, Switzerland) and results used to concentration-standardise the urine FRAP values. Urine hydrogen peroxide was measured by an in-house modified microplate method.
2.3.4 Ferric Reducing /Ascorbic Acid (FRASC) Assay

Reagents and equipments used for FRASC were as described in detail elsewhere using Cobas Fara centrifugal analyzer (Roche Diagnostics, Basel, Switzerland). FRASC reagents were as follows: 300 mM acetate buffer, pH 3.6, prepared by dissolving 3.1 g sodium acetate trihydrate (Riedel-de Haen, Hannover, Germany) in distilled water with 16 ml glacial acetic acid (BDH Laboratory Supplies, Poole, England) added and make up to 1 L with distilled water; 10 mM TPTZ (2,4,6 tripyridyl-s-triazine; Fluka Chemicals, Buchs, Switzerland) solution in 40 mM HCL (BDH Laboratory Supplies); 20 mM FeCl$_3$.6H$_2$O (BDH Laboratory Supplies) solution in distilled water. Working FRASC reagent was prepared as needed by mixing 20 ml acetate buffer with 2 mL of TPTZ solution and 2 ml of ferric chloride solution. A 4 IU/ml solution of ascorbic oxidase (Sigma Chemical, St Louis, MO) was prepared in distilled water, aliquoted, and stored at -70°C until needed.

For FRASC analysis, 100 µl of each sample, (calibrators, control or sample) were added into each of two sample cups. To one of the paired cups, 40 µl of ascorbic oxidase solution was added; 40 µl of distilled water was added to the other cup. After mixing, the paired ascorbic oxidase-diluted (+ao) and distilled water-diluted (-ao) samples were loaded onto the Cobas Fara centrifugal analyzer for automatic reagent/sample mixing and measurement of the changes in absorbance at 593 nm (ΔA$_{593\text{ nm}}$) for the 0 to 1 minute reaction time window. Using the paired water-diluted (-ao) and ascorbic oxidase-diluted (+ao) samples, the ascorbic acid concentration was calculated as follows:

\[
\frac{0-1 \text{ min } \Delta A_{593 \text{ nm}} \text{ of the test sample} \times \text{ FRAP value of standard (µM)}}{0-1 \text{ min } \Delta A_{593 \text{ nm}} \text{ of the standard}}
\]
2.3.5 Measurement of plasma alpha tocopherol using HPLC

Alpha tocopherol was measured, with slight modification, following the HPLC procedure of Brandt et al, 1996. Briefly, 100 µl plasma were mixed with 100 µl of ethanol (Riedel de Haen, Seelze, Germany) and 100 µl of 150 µmol/L α-tocopherol acetate (Merck, Darmstadt, Germany) (internal standard). The mixture was then vortex mixed and 200 µl hexane (Fisher Scientific, Loughborough, UK) containing 0.5g/L butylated hydroxytoluene (BHT) (Sigma chemical Company, St Louis, USA) were added to each calibrator or plasma samples and vortexed for 30 seconds. Mixtures were then centrifuged at 2500g for 10 minutes at 4°C. 120 µl of the hexane layer were transferred to a glass test tube and the hexane was evaporated under nitrogen at about 50°C. 60 µl mobile phase were added to dissolve the extracts. Mobile phase was methanol:toluene (Riedel de Haen), 80:20 (v/v). Each calibrator/sample extract was transferred to a small glass vial fitted with a micro glass insert and the vial was placed inside the HPLC autosampler at 4°C. 20 µl of each calibrator were injected into the HPLC system.

The HPLC system used comprised an Alliance 2690 Separations Module with temperature-controlled column chamber (Waters, Milford, MA, USA), Waters 996 Photodiode Array Detector and Waters Millennium PDA software (version 3.05.01), a reversed-phase ISCO C18 analytical column (5 µm, 250 x 5 mm i.d.; ISCO Inc., Lincoln, Nebraska, USA) and a Waters Sentry guard column (Symmetry C18 3.5 µm, 3.9 x 20mm i.d.). Detection was at 292 nm and the flow rate was 1.0 ml/min.
2.3.6 Statistical analysis

For statistical analysis, Graphpad Prism was used (version 3.0, San Diego, USA). The pre-versus post data were analysed using the Wilcoxon matched pairs test to compare response to each treatment. The Mann Whitney test was used to compare responses to Kei Tze and placebo.

2.3.7 Ethical considerations

Kei Tze had no known toxicity, and no health hazards were anticipated in association with the supplementation of the diet with the planned dose of Kei Tze. Anticipated hazards were associated only with collection of venous blood, but this be taken under standard, safe, protocols by an experienced phlebotomist and using new, sterile, disposable needles and syringes.

There may be no benefits to the volunteers, although Kei Tze was reported to tonify the kidneys and promote the production of essence, to strengthen the legs, to nourish the liver and brighten the eyes. The study would, however, add to scientific knowledge in relation to absorption of Kei Tze antioxidants, All information related to each participant remained confidential, and would be identifiable by codes only know to the researcher. No volunteer would be identified or identifiable from presented or published data.

The University Human Subjects Ethics Committee approved this study, and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000.
3. Results

3.1 In vitro studies of total antioxidant power in different brands of Kei Tze products

Results for FRAP in different extraction medium of the four brands showed a wide range (Table 3 & Figure 10). The Kei Tze extracts contained significant antioxidant capacity, with FRAP values ranging from 25.35 μmol/g to 373.53 μmol/g. In 100% ethanol, Kei Tze extracts showed the lowest FRAP values compared with their corresponding FRAP values in boiling distilled water, acetate buffer pH 3.6 and distilled water at room temperature. Results on acidic extracts of Kei Tze were markedly higher than those of water extracts at room temperature. In boiling water, FRAP values of different brands of Kei Tze were generally higher (except for NingXia brand) than those of water extracts at room temperature. Natural Wolfberry brand in boiling water showed the highest FRAP value with 373.53 μmol/g of Kei Tze. Rich Nature™ Wolfberry brand showed the second highest FRAP value with 318.93 μmol/g of Kei Tze. Ascorbic acid content of the four brands of Kei Tze tested was low, and ranged from 0.15 to 2.37 μM/g of Kei Tze. Because of this, the FRAP values of the berries was measured (after preliminary trials by FRASC) using the original FRAP assay in order to make the measurements as precise as possible. For the FRAP assay in this in-vitro study, precision was excellent: within-run coefficients of variation (CVs) were < 1.0% at 920 μM and 2200 μM and the between-run coefficients of variation (CVs) were < 1.2% at 920 μM and 2200 μM. For FRASC assay, within-run coefficients of variation (CVs) and between-run coefficients of variation (CVs) were < 4.2% at 920 μM and 2200 μM.
FRAP values of Kei Tze in different extraction medium

Figure 10  Total antioxidant power (expressed as FRAP value/μM) of 10% w/v Kei Tze extracts; results are mean of triplicate readings.

On the basis of the FRAP value alone, Natural Wolfberry brand would be selected as the supplement for the in-vivo supplementation study because it showed the highest FRAP value in boiling water. However, for the safety purpose to act as a food supplement, Kei Tze with good quality control should be chosen for in-vivo supplementation. Finally, Rich Nature™ Wolfberry brand was chosen because it has a detailed quality control documentation, which covered analysis of nutrients, micro-bacteria, pesticides and heavy metals, as well as having high antioxidant content. This analysis report ensured the standard of Kei Tze was suitable for supplementation in human.
FRAP value of Kei Tze (results are mean of triplicate readings)

<table>
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<th>Product</th>
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<th>Acetate buffer pH 3.6</th>
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<td></td>
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</tr>
<tr>
<td>Rich Nature™ Wolfberry</td>
<td>3189.31</td>
<td>132.30</td>
<td>3212.25</td>
<td>32.82</td>
</tr>
<tr>
<td>Natural Wolfberry</td>
<td>3735.39</td>
<td>147.73</td>
<td>3554.18</td>
<td>35.91</td>
</tr>
</tbody>
</table>

Table 3: Total antioxidant power (expressed as FRAP value/μM) of 4 different brands of 10% w/v Kei Tze extracts.

3.2 Set up and evaluation of HPLC method to measure lutein and zeaxanthin

Figure 11 - 13 shows the chromatograms corresponding to, respectively, pure lutein standard, pure zeaxanthin standard and mixed standard containing lutein, zeaxanthin and carotene. The peaks for lutein, zeaxanthin and carotene were well resolved, with retention times 10.3, 10.7 and 55.0 minutes respectively. Figure 14 showed the chromatogram of normal pooled plasma. Peaks for lutein, zeaxanthin and carotene can be identified in the normal pooled plasma by comparing peak retention times to those of known standard.
Figure 11  Chromatogram of a pure (2 μg/ml) lutein standard detected at 450 nm, 2.5 μl was injected at flow rate 1.0 ml/min. Retention time of lutein was 10.133 minutes.

Figure 12  Chromatogram of a pure (2 μg/ml) zeaxanthin standard detected at 450 nm, 2.5 μl was injected at flow rate 1.0 ml/min. Retention time of zeaxanthin was 10.762 minutes.
Figure 13  Chromatogram of mixed lutein, zeaxanthin and carotene (each 1 μg/ml) detected at 450 nm, 5 μl was injected at flow rate 1.0 ml/min from 0 to 12 minutes and at 2.5 ml/min from 13 to 60 minutes. Retention time of lutein zeaxanthin and β-carotene were 10.3, 10.7 and 55.0 minutes respectively.

Figure 14  Chromatogram of normal pooled plasma detected at 450 nm, 25 μl was injected at flow rate 1.0 ml/min from 0 to 12 minutes and at 2.5 ml/min from 13 to 60 minutes. Retention time of lutein and zeaxanthin were 10.2 minutes and 10.6 minutes respectively.
3.2.1 Precision and recovery studies

Precision for plasma lutein and zeaxanthin using this new HPLC method was good: within-day coefficients of variation (CVs) were 7% at 0.2 µg/ml (or 0.35 µmol/L) of lutein and 3% of 0.50 µg/ml (or 0.87 µmol/L) of lutein (n = 6). For plasma zeaxanthin, within-day coefficients of variation (CVs) were 8% at 0.1 µg/ml (or 0.18 µmol/L) and 4% at 0.25 µg/ml (or 0.44 µmol/L) (n = 6). Between-day coefficients of variation (CVs) were 9% at 0.2 µg/ml (or 0.35 µmol/L) of lutein and 5% at 0.40 µg/ml (or 0.70 µmol/L of lutein) (n = 6). For plasma zeaxanthin, between-day coefficients of variation (CVs) were 7% at 0.1 µg/ml (or 0.18 µmol/L) and 6% at 0.20 µg/ml (or 0.35 µmol/L) (n = 6).

Recovery was good. The recovery of lutein in plasma was 96.9 % and the recovery of zeaxanthin in plasma was 96.1% (n = 4) when the normal pooled plasma was spiked with low level of mixed standard containing 15 ng of lutein and 5 ng of zeaxanthin. The recovery of lutein in plasma was 84.8% and the recovery of zeaxanthin in plasma was 83.7% (n = 4) when the normal pooled plasma was spiked with high level of mixed standard containing 60 ng of lutein and 20 ng of zeaxanthin.

3.2.2 Linearity and detection limit

The calibration curves over the range from 0 – 0.5 µg/ml or (0 – 0.88 µmol/L) of lutein (r = 0.9979) and 0 – 0.25 µg/ml or (0 – 0.44 µmol/L) of zeaxanthin (r = 0.9985) shown in Figure 15 and Figure 16 respectively. The linearity between 0 – 2.0 µg/ml or (0 – 3.52 µmol/L) of lutein (r = 0.9995) and the linearity of zeaxanthin between 0 – 1.0 µg/ml or (0 – 1.75 µmol/L) (r = 0.9998) were checked to be linear as shown in Figure 17 and Figure 18 respectively. The minimum detectable limits for lutein and zeaxanthin were each 0.3 ng. The detection limit was defined as the lowest amount of the analyte in the standard that could be
detected from zero, and was calculated as mean +3SD of ten blank readings.

3.2.3 Lutein and zeaxanthin content in Kei Tze

The content of lutein and zeaxanthin in Kei Tze (Rich Nature™ Wolfberry brand) used in our supplementation trial were determined. After hydrolysis of Kei Tze extract, the concentration of lutein and zeaxanthin in Kei Tze was 5.8 µg/g and 194 µg/g of dried Kei Tze berry respectively.
Figure 15  Calibration curve for lutein standard

(Each point is mean of four readings n = 4)

Figure 16  Calibration curve for zeaxanthin standard

(Each point is mean of four readings n = 4)
Figure 17  Linearity check for lutein standard
(Each point is mean of duplicate readings)

Figure 18  Linearity check for zeaxanthin standard
(Each point is mean of duplicate readings)
3.3 28 days in vivo supplementation of Kei Tze with a single-blinded, placebo-controlled human intervention of parallel design

A total of 27 apparently healthy Chinese were entered into the in vivo 28 days supplementation trial of Kei Tze. Information on subjects is summarized in Table 4. Subjects were allocated on non-selective basis into either study group (n = 14) or control group (n = 13). The overall male to female ratio for all the subjects was 1 to 2.86. Male to female ratio was 1 to 6 and 1 to 1.6 in the study group and the control group respectively. The age range of the all subjects was 18 to 48 years (mean (SD) 27.6(9.46)) and BMI of 17.9 to 24.7 kg/m² (mean (SD) 20.88(2.2)). The age range of the subjects was 18 to 44 years (mean (SEM) 28.0(2.68)) and 18 to 48 years (mean (SEM) 27.2(2.54)) in the study group and the control group respectively. The age distribution of all subjects in this supplementation study was shown in Figure 19. Age distribution in control group was similar to those in study group, which made results more comparable.

There were a total of two visits for each subject in this supplementation study. A total of 27 subjects entered into the study and they were divided into 4 groups (each group containing 6 – 7 volunteers). Each group came to the laboratory in the School of Nursing, HKPU as our time schedule. The overall attendance was excellent: 100% in the first visit and 92.5 % in the second visit. The overall compliance of all subjects in this in-vivo study was 92.5% as only two subjects in the control group withdrew at the end of the trial because of their own personal matters. One subject has left Hong Kong to study abroad and another subject could not attend the second visit because she needed to sit for her final examination.
<table>
<thead>
<tr>
<th></th>
<th>Study Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Number of females</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>Number of males</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Age Range (years)</td>
<td>18 - 44</td>
<td>18 - 48</td>
</tr>
<tr>
<td>Age, [Mean, (SEM)] years</td>
<td>28.0 (2.68)</td>
<td>27.2 (2.54)</td>
</tr>
<tr>
<td>Height/m, [Mean, (SEM)]</td>
<td>1.59 (0.016)</td>
<td>1.62 (0.025)</td>
</tr>
<tr>
<td>Weight/kg, [Mean, (SEM)]</td>
<td>51.39 (1.76)</td>
<td>57.68 (2.92)</td>
</tr>
<tr>
<td>Body Mass Index, [Mean, (SEM)]</td>
<td>20.2 (0.55)</td>
<td>21.61 (0.60)</td>
</tr>
</tbody>
</table>

Table 4  Information of subjects collected from questionnaire.

Figure 19  Age distribution of subjects in study group (n = 14) and control group (n = 13)
In this in-vivo supplementation study, changes in fasting plasma, urine and saliva variables before and after 28 days of Kei Tze supplementation are shown in Table 5. No significant increase in plasma FRAP values in the study group after 28 days Kei Tze supplementation were seen, however, it can be seen that the FRAP values in plasma were significantly (P<0.05) lower in the placebo group, as compared to the study group, at the end of the study. No significant differences were seen in urine FRAP and saliva FRAP. For plasma ascorbic acid, no significant change was seen after Kei Tze supplementation. There was a significant (P<0.05) increase of total plasma α-tocopherol in the study group after 28 days Kei Tze supplementation. However, no significant change of plasma α-tocopherol was seen after lipid-standardisation. Plasma uric acid was significantly (P<0.05) lower in the placebo group after 28 days Kei Tze supplementation. No significant change was seen in erythrocyte SOD activities. Whole blood GPx activity tended to be higher after 28 days Kei Tze supplementation, but changes were not statistically significant. Urine H₂O₂ tended to be lower after Kei Tze supplementation but again, changes were not statistically significant.

For plasma lutein, the response to Kei Tze in the study group was not significantly different from response to placebo after 28 days Kei Tze supplementation showed in Table 6. However, for plasma zeaxanthin, there was a significant increase (p<0.01) for the post-supplementation sample in the study group. Response to Kei Tze in the study group was significantly different (p<0.01) from the response to placebo. After lipid standardization, plasma zeaxanthin was still significantly increased (p<0.01) in the study group. Response to Kei Tze in the study group was significantly different (p<0.01) from response to placebo after 28 days Kei Tze supplementation. Lutein and zeaxanthin results are present in both μg/L and μmol/L to make comparison with published literature (often in μg/L) easier.
Table 5  Values of test results [mean (SEM)] in fasting samples (study group n = 14, control group n = 11) before and after 28 days supplementation with Kei Tze and placebo

<table>
<thead>
<tr>
<th>Fasting blood sample</th>
<th>Placebo</th>
<th>Response to placebo</th>
<th>Kei Tze</th>
<th>Response to Kei Tze</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td><strong>Plasma FRAP (μmol/L)</strong></td>
<td>1101(39)</td>
<td>966(49)</td>
<td>-135(32)*</td>
<td>1061(56)</td>
</tr>
<tr>
<td><strong>Urine FRAP (μmol/mmol creatinine)</strong></td>
<td>0.95(0.06)</td>
<td>0.96(0.06)</td>
<td>0.01(0.04)</td>
<td>1.04(0.09)</td>
</tr>
<tr>
<td><strong>Saliva FRAP (μmol/L)</strong></td>
<td>608.6(112)</td>
<td>649.0(107)</td>
<td>41.1(76)</td>
<td>601.8(67)</td>
</tr>
<tr>
<td><strong>Plasma ascorbic acid (μmol/L)</strong></td>
<td>60.6(7.6)</td>
<td>58.9(6.0)</td>
<td>-1.78(3.5)</td>
<td>60.2(4.8)</td>
</tr>
<tr>
<td><strong>Plasma α-tocopherol (μmol/L)</strong></td>
<td>26.04(2.5)</td>
<td>25.74(2.2)</td>
<td>-0.30(0.7)</td>
<td>21.58(1.5)</td>
</tr>
<tr>
<td><strong>Lipid Standardised α-tocopherol (μmol/mmol TC+Tg)</strong></td>
<td>4.52(0.3)</td>
<td>4.65(0.2)</td>
<td>-4.23(13.5)</td>
<td>4.08(0.2)</td>
</tr>
<tr>
<td><strong>Plasma total cholesterol (mmol/L)</strong></td>
<td>4.66(0.3)</td>
<td>4.31(0.2)</td>
<td>-0.34(0.2)</td>
<td>4.63(0.3)</td>
</tr>
<tr>
<td><strong>Plasma triacylglycerol (mmol/L)</strong></td>
<td>1.06(0.2)</td>
<td>1.21(0.2)</td>
<td>0.14(0.2)</td>
<td>0.67(0.1)</td>
</tr>
<tr>
<td><strong>Plasma uric acid (umol/L)</strong></td>
<td>296(22.6)</td>
<td>228(15.2)</td>
<td>-68(11.2)*</td>
<td>275(23.2)</td>
</tr>
<tr>
<td><strong>Erythrocyte SOD (units/g Hb.)</strong></td>
<td>1290(48)</td>
<td>1301(68)</td>
<td>11(51)</td>
<td>1319(64)</td>
</tr>
<tr>
<td><strong>Whole blood GPx (units/L)</strong></td>
<td>8260(384)</td>
<td>8748(493)</td>
<td>488(363)</td>
<td>7455(1316)</td>
</tr>
<tr>
<td><strong>Urine H_{2}O_{2} (μmol/mol creatinine)</strong></td>
<td>315(76)</td>
<td>388(88)</td>
<td>76(87)</td>
<td>449(190)</td>
</tr>
</tbody>
</table>

* Significantly different from response to placebo, P<0.05 (Mann Whitney test).
Table 6  
Values of plasma lutein and plasma zeaxanthin [mean (SEM)] in fasting samples (study group n = 14, control group n = 11) before and after 28 days supplementation with Kei Tze and placebo

<table>
<thead>
<tr>
<th>Fasting blood sample</th>
<th>Placebo</th>
<th>Response to placebo</th>
<th>Kei Tze</th>
<th>Response to Kei Tze</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>Plasma zeaxanthin (µg/L)</td>
<td>214(21.1)</td>
<td>246(19.8)</td>
<td>31(21.3)</td>
<td>217 (19.4)</td>
</tr>
<tr>
<td>Plasma zeaxanthin (µmol/L)</td>
<td>0.37 (0.04)</td>
<td>0.43(0.03)</td>
<td>0.05(0.03)</td>
<td>0.38 (0.03)</td>
</tr>
<tr>
<td>Lipid standardised plasma zeaxanthin</td>
<td>0.065</td>
<td>0.078</td>
<td>0.012</td>
<td>0.078</td>
</tr>
<tr>
<td>(µmol/mmol TC+Tg)</td>
<td>(0.007)</td>
<td>(0.008)</td>
<td>(0.007)</td>
<td>(0.007)</td>
</tr>
<tr>
<td>Plasma lutein (µg/L)</td>
<td>817(118)</td>
<td>963(158)</td>
<td>146(89)</td>
<td>930(119)</td>
</tr>
<tr>
<td>Plasma lutein (µmol/L)</td>
<td>1.43(0.20)</td>
<td>1.69(0.27)</td>
<td>0.25(0.15)</td>
<td>1.63 (0.21)</td>
</tr>
<tr>
<td>Lipid Standardised plasma lutein</td>
<td>0.25(0.04)</td>
<td>0.30(0.05)</td>
<td>0.04(0.02)</td>
<td>0.33(0.04)</td>
</tr>
<tr>
<td>(µmol/mmol TC+Tg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different from response to placebo, P<0.01 (Mann Whitney test).
4. Discussion

4.1 In vitro studies of total antioxidant power in different brands of Kei Tze products

Our data confirmed Kei Tze has antioxidant capacity. The Kei Tze extracts contained significant antioxidant capacity, with FRAP value ranging from 25.35 µmol/g to 373.53 µmol/g of dried Kei Tze berry. Comparing the antioxidant content of teas, fruits and vegetables (Benzie & Szeto, 1999a; Szeto et al, 2002), the FRAP value of black tea ranged between 132 - 654 µmol/g and green tea between 272 - 1144 µmol/g of dry tea leaves, the FRAP value of fruits and vegetables was 81 µmol/g for orange, 70 µmol/g for green apple, 39 µmol/g for onion, 38 µmol/g for cauliflower. For pure ascorbic acid, the FRAP value was 11364 µmol/g. The FRAP value of Lingzhi was 360 µmol/g (Wachtel-Galor et al, 2004).

The highest FRAP value of Kei Tze was 373.5 µmol/g, indicating a fairly high antioxidant content. However, what was of special interest to us was whether these antioxidant compounds can be absorbed and passed into the plasma, and whether they caused discernible effect on biomarkers of antioxidant status, oxidative stress or risk of age-related disease after a period of supplementation. This leads us to the present investigation on planning a 28 days in-vivo supplementation of Kei Tze to address the above questions.

Our data showed that different brands of Kei Tze in the market have various level of antioxidant capacity. Such variation in antioxidant capacity may reflect the "quality" of Kei Tze. The quality of Kei Tze and other plant-based foods is affected by different factors such as cultivation method, temperature, humidity, region of plant and storage method etc. Even though the four brands of Kei Tze in our in-vitro studies showed some difference in their antioxidant power, all of them still contained high content of antioxidant capacity, indicating they may act as a good source of dietary antioxidants. As there has not been much research data on the study of antioxidant power of in Kei Tze, the present data in our in vitro-studies can serve act as good reference for dietary plan.
Our results also revealed that the Kei Tze extracts in different extraction medium showed different antioxidant power. Generally, the FRAP value of Kei Tze extract was higher in water-based preparation than in ethanol-based preparation. It indicated that most of the antioxidants in Kei Tze were rather water-soluble. FRAP values of Kei Tze extracts in boiling water or acidic pH 3.6 were generally higher than those in cold water. This reflects that the mode of preparation can greatly affect the antioxidant power of Kei Tze. For most Chinese people, traditional cooking habit is to boil Kei Tze with meat in preparing soup. It seemed that the antioxidants in Kei Tze were more extractable in boiling water by using this cooking method.

The results of in vitro-studies can serve as a good guide for us to decide which brand of Kei Tze would be used as supplement in supplementation trial. It also provided an objective reference for us to decide the mode of Kei Tze preparation used in supplementation. Based on our in vitro data and careful consideration about the safety of different products for food supplementation, Rich Nature™ Wolfberry brand was finally chosen because it had a detailed quality control documentation, which covered analysis of nutrients, micro-bacteria, pesticides and heavy metals, as well as having high antioxidant content. This good quality control report ensured the standard of Kei Tze was suitable for supplementation in human. Berries were put into boiling water because, on the basis of our in vitro data, the antioxidant content of the tea was optimized. As there was no published reference for the daily dosage of Kei Tze for supplementation, we simply followed the recommended daily dosage of Kei Tze (15 g each day) from the instructions of the producer. Thus, all the conditions have been optimized and were suitable for in vivo supplementation in human.

Basically, the four tested Kei Tze products can be broadly divided into two categories. For Quality Fructus Lycii (Yue Hwa Chinese Product Ltd, HK) and NingXia Kei Tze (local herb store), they were sold as raw herb for making soup or used in herbal formula. These two
brands of products were washed and cooked as usual in our menus. For Natural Wolfberry (Organic Garden Ltd, HK) and Rich Nature™ Wolfberry (Rich Nature Labs Inc. USA), they were sold as snack or health supplement, which were ready for eating. It is only in recent years that the producers of Kei Tze have started to prepare and pack their Kei Tze products as snack or health supplements and sold in the Hong Kong market. Thus, we realized that the culture of consuming Kei Tze as a health supplement is becoming more popular in Hong Kong.
4.2 Set up and evaluation of HPLC method to measure lutein and zeaxanthin

In spite of the large number of dietary carotenoids absorbed into the blood circulation, lutein and zeaxanthin are only present in the human macula. The specific location of zeaxanthin-lutein in human retinas has drawn speculation on the possible protective benefit of these two carotenoids against age-related macular degeneration. Although Kei Tze has been widely used by the Chinese for centuries with an expected health benefit to the eye, the mechanism of its effect was not known. A common problem in understanding the function of this herbal medicine was the absence of a suitable bioassay to identify the active agents. In the past, scarce research was conducted for measuring plasma lutein and zeaxanthin using HPLC. Even though a variety of HPLC methods have been developed for the measurement of carotenoids in human plasma, most of the methods were either not capable of measuring both lutein and zeaxanthin, or required a complex elution systems for their determination in plasma. As lutein and zeaxanthin are structural isomers with the same molecular formula (C_{40}H_{56}O_{2}), they often elute as a single peak during HPLC analysis in published papers. Because of the above reason, their complete separation or measurement by HPLC may not be so attractive for researcher. However, the specific accumulation of lutein-zeaxanthin in human retina has drawn our special interest on the possible protective benefit of these two carotenoids against AMD. As Kei Tze was reported to contain a high content of zeaxanthin, which may be good for our vision, setting up of new HPLC method for measurement of lutein and zeaxanthin became essential in this supplementation trial. It provided a good biomarker tool for our in-vivo supplementation to assess the pre and post samples changes in plasma lutein and zeaxanthin or for measuring the content of lutein and zeaxanthin in Kei Tze.

From the experience of setting the present HPLC method, several technical considerations have been concluded for the assay. Extraction was an essential step in this
assay. For complete extraction of lutein and zeaxanthin, some critical points such as the ratio of volumes, temperatures and mixing times should be optimized. Aebischer et al, 1999 reported that the optimum water to plasma ratio was 1 for carotenoid in the extraction procedures. In our extraction procedure for lutein and zeaxanthin, plasma was first diluted with water containing 0.1% ascorbic acid in 1:1 ratio, and then the proteins should be precipitated with equal volume of ethanol containing 0.1% BHT to ensure a complete deproteinization. Incomplete protein precipitation may cause an un-breakable gelly emulsion layer if the extracting solvent, hexane, was in large quantity with vigorous shaking. This gelly emulsion layer greatly reduced the yield of extraction of lutein and zeaxanthin into the hexane layer, giving an unsatisfactory, low result. The rolling time for extraction procedure was extended to 30 minutes because insufficient rolling time also resulted in very low recoveries of both lutein and zeaxanthin. Thus, all the above conditions were optimized.

Both lutein and zeaxanthin were reported freely soluble in tetrahydrofuran (THF) (Talwar et al, 1998). Their pure solids were best first prepared in THF to ensure complete dissolution. However, THF were unstable and sensitive to oxygen. Addition of BHT can stabilize THF from oxidation during storage. Hence, addition of little THF/BHT together with the mobile phase to reconstitute the dry extracts was essential, otherwise the results will be very low.

Lutein and zeaxanthin seemed to be labile to light and oxygen as both molecules contained long chains of conjugated double bonds. To minimize the degradation due to these factors, preparations of standards and plasma samples were avoided carrying out in strong light and evaporating of extracts was not under air. We used two antioxidants in our assay, 0.1% ascorbic acid in aqueous phase and 0.1% BHT in non-aqueous phase, to protect carotenoids against oxidative losses during sample processing and analytical procedures. Aluminium foil was used to protect the sample from direct illumination of light, which may
cause degradation of carotenoid. To minimize the isomerisation of carotenoids, which may manifest as a broadening of the peaks with reduction in peak height, the use of ascorbic acid was reported to facilitate the drying of hexane extracts at a higher temperature (40°C). This can shorten the drying time to dry the sample. Hence the use of ascorbic acid and keeping the sample in iced cold water between preparation and analysis allowed us to analyze a larger number of samples without detectable isomerisation of carotenoids.

From the chromatogram of normal pooled plasma, it was found that plasma lutein and zeaxanthin eluted at retention times 10.2 and 10.6 minutes, respectively. During the setting of the HPLC method, we noted that one of the plasma extractants, β-carotene, eluted out very slowly (more than 2 hours at a flow rate of 1.0 mL/min) under this solvent system. As this β-carotene peak may interfere and overlap the peaks of lutein and zeaxanthin in the subsequent samples during analysis, giving falsely high results, sample injection intervals must be long enough to allow column 'washout' of carotene. Hence, the flow rate was time-programmed to increase to 2.5 mL/min once lutein and zeaxanthin were eluted. With the current program, β-carotene gave an eluted peak at around 55 minutes. Hence, the running time of each sample injection was finally set to 1 hour in order to avoid the interference caused by the β-carotene peak. As our HPLC system was an auto-sampling system, it was convenient for us to perform tests in batches, even though the running time for each injection was 1 hour.

From the results of recoveries, it seems that high level test gave lower recovery (about 84%), indicating possibly incomplete extraction or greater loss at high level. Assuming that the degradation is negligible (as antioxidative protection by ascorbic acid in aqueous phase and by BHT in non-aqueous phase, sheltering from strong light), one possible reason of low recovery was that single extraction by the extracting solvent, hexane, was not efficiently enough for complete extraction and it probably needs to repeat another extraction. Another reason may be due to incomplete re-constitution of the dried extracts that were remaining in
the wall of the drying tube though addition of little THF (with BHT) before mobile phase (the re-constituting solvent) had been attempted to reduce this loss. Finally, loss may also occur during nitrogen blowing if its pressure is too high. More tests are hence recommended to modify the extraction method so as to improve the recovery. However, the use of internal standard (For example, lutein monohexyl (or monopropyl) ether, β-apo-8’-carotenyl myristate or echinenone), if available, can minimize all these mentioned effects of extraction, evaporation and re-constitution, resulting in increasing both precision and accuracy of the assay.

Most of the reverse-phase HPLC methods used gradient conditions to assay lutein/zeaxanthin. To save and economise the consumption of the mobile phase, isocratic, instead of gradient, condition was set up to recirculate the solvent in this study. Our present isocratic HPLC method established here was simple, analytically reliable and capable of simultaneously measuring lutein and zeaxanthin in human plasma. The chromatography was highly efficient resulting in sharp and well-resolved peaks, which was suitable for routine use in laboratory.

Not many papers have been published on human plasma levels of zeaxanthin. Most of the papers gave the level of lutein only or as the total of lutein and zeaxanthin (since both analytes co-eluted in many reverse-phase solvent system). The reference intervals for lutein and zeaxanthin in human serum are reported to be 50 – 250 μg/L or (0.088 – 0.440 μmol/L) and 8–80 μg/L or (0.014 – 0.140 μmol/L) respectively (ARUP Laboratories). Another reference was from Aebscher et al, 1999 using normal-phase HPLC: human plasma lutein level was 336 μg/L or (0.591 μmol/L) and zeaxanthin level was 89.3 μg/L or (0.157 μmol/L). In our in-vivo supplementation study, the mean (SEM) of plasma lutein and zeaxanthin in the study group were 930(119) μg/L or 1.63 (0.21) μmol/L and 217(19.4) μg/L or 0.38(0.03) μmol/L respectively. The mean (SEM) of plasma lutein and zeaxanthin in the control group
were 817(118) μg/L or 1.43(0.20) μmol/L and 214(21.1) μg/L or 0.37(0.04) μmol/L respectively. Compared with the above results with the reference from Aebischer et al, 1999, the plasma lutein and zeaxanthin in our subjects were much higher (2.5 times) than the reference levels in the previous studies quoted. We suggested the difference of plasma lutein and zeaxanthin may be due to the dietary habit in different population. For most of the Chinese, they generally liked to eat more fruits and vegetables rather than meat. However, the reason accounted for the high plasma lutein and zeaxanthin in our subjects needed further research to study.

The content of lutein and zeaxanthin in Kei Tze (Rich Nature™ Wolfberry) used in our supplementation trial were determined. After hydrolysis of Kei Tze extract, the concentration of lutein and zeaxanthin in Kei Tze was determined to be 5.8 μg/g and 194 μg/g of dried Kei Tze berry respectively. Comparing with previous paper published, the content of zeaxanthin in Kei Tze was reported to range from 11.5 to 43.3 μg/g of Kei Tze (Lam et al, 1999). Our present data showed that the content of zeaxanthin in our supplement (Rich Nature™ Wolfberry) was 4.5 times higher than the content of zeaxanthin published in the previous literature. A more recent published paper reported that their chromatographic studies of Kei Tze indicated a high content of zeaxanthin (300 μg/g) (Leung et al, 2001). Nevertheless, the dimension of zeaxanthin content in Kei Tze in all studies findings was similar. However, the difference of the zeaxanthin content in different Kei Tze may be explained by the quality or grading of different Kei Tze products. In fact, few published data exist on the content of lutein and zeaxanthin in Kei Tze. The composition of different types of carotenoid in Kei Tze also has not been reported previously.

Our data confirmed that Kei Tze was an excellent dietary source for zeaxanthin. In the study group, we estimated that each subject took 87 μg lutein and 2910 μg zeaxanthin in each day in the Kei Tze. The data also showed that the measured amount of lutein content is
negligible compared with the amount of zeaxanthin in Kei Tze. The natural occurrence of the ester form of zeaxanthin accounts for the stability of the medicinal effect of Kei Tze in the dried berries (Zhou et al, 1999). It was because the hydroxyl groups in zeaxanthin can be damaged by oxidative degradation. Acylation of zeaxanthin should prevent oxidation of hydroxyl groups. Thus, the occurrence of these remarkably high amounts of zeaxanthin ester in Kei Tze was a fascinating natural design to provide one stable source of zeaxanthin in a small berry for a health benefit to our eyes.
4.3 28 days in vivo supplementation of Kei Tze with a single-blinded, placebo-controlled human intervention of parallel design

*Fructus Lycii* is a small red berry commonly used in home cooking in Hong Kong and many parts of Asia because of its flavour as well as general health benefits. It is also used in traditional Chinese herbal medicine for the improvement on visual acuity. In recent years, a variety of commercial Kei Tze products were available in the form of raw material for making soup, snack, capsule and dietary supplements. However, the problem facing consumers and healthcare professionals is that there are insufficient scientific data available with regard to the efficacy, safety and mode of action of Kei Tze. This underlines the urgent need for extensive research and controlled supplementation trials to support their use as food supplements for visual health promotion.

For the recruitment of volunteers in our supplementation trial, we targeted on recruiting convenience subjects as much as possible. We started to recruit subjects at the beginning of February, 2003 and planned to start the supplementation at the beginning of April, 2003. However, the supplementation was postponed because of the outbreak of Severe Acute Respiratory Syndrome (SARS) in March, 2003. It was not until the end of May, 2003 that the project could be resumed. To a certain degree, this incident gave some impact on the project. It was because some of the original volunteers refused to participate in the supplementation trial. Most of the reasons for their refusal were due to the incidence of SARS. They felt psychologically uncomfortable because there were two visits for blood collection. After our clear explanation for safety of blood taking procedures, volunteers accepted to participate the supplementation trial again. Finally, there were 27 subjects entered into this in vivo supplementation study, which started in the mid of August, 2003 and ended at the end of September, 2003. For the design of this supplementation trial, we used a single-blinded, placebo-controlled human intervention of parallel design instead of crossing-over
design. There was limitation for using crossing-over design because of the time limit for this project. The wash out period for the subjects may be long after the crossing-over between the study and placebo group.

In our in vitro studies of Kei Tze, our data confirmed that Kei Tze has fairly high antioxidant capacity and high content of zeaxanthin. However, what was interesting and which has not been previously studied was the objective measure of these antioxidant capacity and plasma zeaxanthin, whether these antioxidant compounds can be absorbed and passed into the plasma, and whether they caused any discernable effect on biomarkers of antioxidant status, oxidative stress or risk of age-related disease after a period of supplementation was still unknown.

According to the literature and internet search, there is no agreed recommended dosage of Kei Tze. In summary, most of the manufacturers of different Kei Tze products recommend a dosage ranged between 5 g to 20 g a day of a dried Kei Tze. However, dose of up to 50 g a day has been used with no reported toxic side effects (Gan et al, 1992). The dosage used in our supplementation study was single daily dose of 15 g of dried Kei Tze prepared in boiling water for 28 days. This dose was aligned within the recommended dose from Rich Nature™ Wolfberry manufacturer for health promotion and was suitable for this first supplementation trial of healthy subjects. According to the follow-up results of our subjects, only one subject reported constipation after she started supplementation for 7 days. However, she reported that she usually needed to work overtime in her office and did not often take her regular dinner. We suggested her to take her meal regularly and ate more fruits and vegetables in her diet. Finally, the problem of constipation resolved after a few days, and this problem was not considered to be due in any way to her participation in the trial.

Our in-vivo supplementation data showed that 28 days supplementation with Kei Tze maintained the baseline plasma antioxidant capacity (expressed as FRAP) and uric acid in the
study group after the supplementation study. No significant change was found for urine FRAP and saliva FRAP before and after the supplementation. However, it was of interest that the plasma FRAP values and uric acid significantly (p<0.05) decreased after placebo ingestion in the control group. Although uric acid is regarded as an important endogenous antioxidant, high uric acid is associated with increased risk of CHD (Benzie & Strain, 1996). It is not clear why uric acid decreased in the placebo group, but possibly this was due to personal dietary/seasonal changes in these individuals, and this could not be completely controlled for in this type of study. As uric acid makes up 60% of total plasma FRAP a decrease in uric acid would cause a decrease in FRAP.

Regular adequate intake of dietary antioxidants is necessary to maintain defences to deal with continued exposure to oxidative stress from the environment. Thus, replenishment of plasma antioxidant buffer system by diet was essential to maintain our health. Once deficient in any of dietary antioxidant, the decreased plasma antioxidant capacity may be unable to meet with the continued oxidative challenge, and finally may result in disease status.

The present data indicated that the Kei Tze preparation used in our 28 days in-vivo study contained a high content of zeaxanthin and at least some of this antioxidant component reached the plasma, and caused a significant increase in plasma zeaxanthin. Even though no statistically significant changes were seen in any of other variables measured, such as plasma ascorbic acid, lutein, SOD, GPx and urine H₂O₂, the pattern of response was interesting. Whole blood GPx concentration tended to increase and urine hydrogen peroxide tended to decrease in the study group after 28 days Kei Tze supplementation. As the level of hydrogen peroxide in urine has been suggested as a potential biomarker of whole body oxidative stress (Yuen et al, 2004), it was believed that the antioxidants in Kei Tze supplement were being absorbed and function well to reduce the oxidative stress in the study group. However, further
study is warranted in the light of our preliminary findings.

The most interesting variable measured was plasma zeaxanthin because there was a 2.5 times increase before and after 28 days Kei Tze supplementation in our study group. Even though we do not know the exact mechanism for this change, we believe that the esterified form of Kei Tze was hydrolysed into free zeaxanthin, and this was absorbed into the blood circulation. In the past, serum and retinal levels of zeaxanthin and lutein were studied after feeding rhesus monkeys an extract of *Fructus Lycii* for 6 weeks (Leung et al, 2001). It was found that serum levels of zeaxanthin and lutein in the feeding group were significantly higher than those of the control group. In particular, the serum zeaxanthin concentration after supplementation with Kei Tze for 6 weeks increased by 2.5 times compared with the basal level. This is the only zeaxanthin absorption study published to date with Kei Tze. Therefore, our data from this controlled human supplementation study provides the first evidence on effect of supplementation with Kei Tze on blood antioxidant status, and specifically changes in plasma zeaxanthin. Our new data showed concordant findings with the monkey model in the previous study, showing 2.5 times increased in plasma zeaxanthin. This significant increase in plasma zeaxanthin has strong implications for delay or prevention of AMD and may benefit health in the long term because the additional supply of zeaxanthin may maintain the macular pigment density. Further study is needed to confirm this, but our data presented here will support this.
Conclusion

In conclusion, results in our in-vitro studies showed that Kei Tze contained antioxidant power. Different brands of Kei Tze products have different levels of antioxidant power. The total antioxidant power of Kei Tze was fairly high in boiling water medium, which suggests this to be a good mode of preparation for its herbal efficiency. The high content of antioxidant capacity in Kei Tze may be a good source of dietary antioxidant. As there has not been much research data on the study of antioxidant power of Kei Tze, the present data in our in vitro-studies can serve act as good reference for dietary plan.

Our isocratic HPLC method established here was simple, analytically reliable and capable of simultaneously measuring lutein and zeaxanthin in human plasma. The chromatography was highly efficient resulting in sharp and well-resolved peaks, which was suitable for using as a good biomarker tool for lutein and zeaxanthin. In our subjects, mean of plasma lutein and zeaxanthin were 2.5 times higher than the reference values used in non-Chinese population. These differences may due to the fact that most of the Chinese like to eat more fruits or vegetable rather than meat, but further work is needed to confirm this. We showed that Kei Tze contained a high content of zeaxanthin (but negligible amount of lutein), indicating it is an excellent dietary source for zeaxanthin for possible maintenance of macular pigment.

In vivo results showed that 28 days supplementation of Kei Tze caused a significant increase (p<0.01) in plasma zeaxanthin of, on average, 2.5 fold. Hopefully, this increase in plasma zeaxanthin may help to prevent AMD by maintaining the macular pigment density, however this remains to be confirmed in future study.

To further our studies in the near future, we propose to recruit more subjects at different age groups for the in vivo supplementation study, which enhance the power of the study for statistical analysis. Instead of using a parallel design in the supplementation study,
we suggest to use crossing-over design for longer supplementation period (3 months), providing with a sufficient wash out period. It is also possible to conduct a double-blinded, placebo-controlled supplementation study by using a new Kei Tze product, W-LBP developed by Rich Nature Labs, in the form of capsule rather than the dried berry. It is because it allows to standard the amount of lutein and zeaxanthin that the subjects are taken, and the dummy may also be available in the form of capsule for the placebo group. We also suggest to collaborate with the Department of Optometry, the Hong Kong Polytechnic University for the measurement of macular pigment density in our subjects in the supplementation trial, which allow us to show the effect of supplementation with Kei Tze on macular pigment density in humans. We hope that with our pilot study data, together with an improved research plan, our future research can input more scientific knowledge to show the herbal effect of Kei Tze on prevention of AMD.
Appendix I

INFORMATION SHEET

EFFECT OF SUPPLEMENTATION WITH FRUCTUS LYCII (KEI TZE) ON BLOOD ANTIOXIDANT STATUS:
IMPLICATIONS FOR AGE-RELATED CHANGES IN MACULAR PIGMENT DENSITY

You are invited to participate on a study conducted by Mr. Cheng Chung Yuen, who is a postgraduate student of the School of Nursing in The Hong Kong Polytechnic University.

The aim of this study is to investigate in vitro study of total antioxidant power of Kei Tze products and to investigate the effect of supplementation of Kei Tze on blood antioxidant status; including total antioxidant power and carotenoids. The study will involve completing a questionnaire, which will take you about half an hour. You will then be asked to take part in a supplementation trial of Kei Tze for 28 days, during which you will be asked to take EITHER two spoonful of Kei Tze berries (which will be supplied) with some warm water (test group), or just warm water (control group), and to provide two one pre- and one post-) fasting blood samples (around 5ml each, which is a teaspoonful, taken around 9am, after an overnight fast and before you have breakfast) from a vein in your arm. We will measure pre- and post-supplementation blood antioxidant status to see if there is any difference between the groups. It is very important that you stick to your regular diet during the 28 days - please do not start taking any other supplements during this time, as this will affect our results.

It is hoped that this information gained from this study will help us understand the disease of age-related macular degeneration in order to develop preventive strategies. The testing should not result in any undue discomfort, but you will need to comply with the schedule for taking Kei Tze daily. All information related to you will remain confidential, and will be identifiable by codes only known to the researcher. You have every right to withdrawn from the study before or during the measurement without penalty of any kind.

If you have any complaints about the conduct of this research study, please do not hesitate to contact Mr. Eric Chan, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in person or in writing (c/o Human Resources Office in Room M1303 of the University).

If you would like more information about this study, please contact Dr. Iris Benzie on tel. no. 27666394 or Mr. Cheng Chung Yuen on tel no. 9163

Thank you for your interest in participating in this study.

Dr. Iris Benzie, Chief Supervisor
Appendix II

Questionnaire 問卷

Name 姓 名：________________________  Sex/Age 性別/年齡：_____________________

Occupation 職業：_____________  Telephone No. 聯絡電話：_____________________

Weight 體重：________ lbs 磅 / __________ kg 公斤

Height 身高：________ ft 呎 __________ inch 吋 / __________ cm 厘米

Smoker 吸煙人仕：Yes 是 / No 否

History of Eye Diseases 眼疾病歷：

1. Glaucoma 青光眼
2. Cataract 白內障
3. Age-related Macular Degeneration 老年黃斑點病變
4. Others 其他 (please specify 請列明) __________________________

Supplement taken 有否進食補充食品：

1. Vitamin caplets 維他命丸 (please specify 請列明) ______________
2. Wolfberry (Kei Tze) 枸杞子
3. Carrot 胡蘿蔔
4. Maize 玉蜀黍
5. Lingzhi 靈芝
6. Others 其他 (please specify 請列明) __________________________

Yes 是 / No 否

Yes 是 / No 否

Yes 是 / No 否

Yes 是 / No 否

Yes 是 / No 否

Yes 是 / No 否
Appendix III  枸杞子抗氧化研究（臨床測試）

枸杞子含有高抗氧化物質，相信有助預防老年黃斑點病變眼疾的作用。透過這次臨床測試，希望有助了解枸杞子對人體中抗氧化指數的影響及其預防疾病的療效。

臨床測試為期 30 天，每位參加者將會在指定的日期及時間於理工大學醫療化驗學系就見兩次（於測試第一天及第三十天）。參加者將被隨機分配為測試組別（Study Group）或對照組別（Control Group）。每次約見會進行血液測試 (抽取 5-8 毫升血液)，收集口腔內細胞，唾液及尿液標本。而每位參加者將被分發 3 塊枸杞子用作臨床測驗。

1) 測試組別的參加者請於每天晚餐後進食 15 克枸杞子。服用方法如下，先將 2 湯匙（相等於 15 克）枸杞子放進杯內，然後沖入約 200-300 毫升熱水，待 10-15 分鐘後，用湯匙輕輕壓碎杯內的枸杞子，然後把泡茶飲用及進食餘下的枸杞子。服用時間為期 30 天。請避免將枸杞子存放在高溫及潮濕地方。多謝合作。

2) 對照組別參加者請於每天晚餐後飲用 200-300 毫升和暖開水。服用時間為期 30 天。已分發的枸杞子將贈送於參加者作日後進食用途，以示謝意。

敬希所有參加者能遵從服用方法進食而無需要改變你的正常飲食習慣，亦不可進食多加的補充食品，例如：維他命丸、大量的胡蘿蔔、玉蜀黍等食品。為確保枸杞子的品質，此臨床測試中所採用枸杞子是經過製造商的嚴格品質測試，符合安全標準。

如對以上飲食方法或測試有任何疑問，歡迎致電 9163 鄭先生（Andy）查詢。
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