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葉綠素衍生物對誘發型 DNA 損傷之保護效應

Preventive Effects of Chlorophyll Derivatives on Induced-type DNA Damage

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中文摘要

葉綠素是植物體中含量最多最廣泛的色素。因此本研究主要探討四種葉綠素的水萃取物，包括脫植醇葉綠素 a 與 b、脫鎂脫植醇葉綠素 a 與 b，對於淋巴球細胞中 DNA 氧化損傷的抗氧化作用；以及對於黃麴毒素所誘導肝細胞 DNA 損傷後的抗基因突變作用。第一部分，將淋巴球細胞分別與四種葉綠素衍生物 5~50μM 共同培養後，再將其暴露在 10 、50μM 的過氧化氫使其誘導氧化傷害。利用單細胞膠體電泳法(彗星分析)觀察 DNA 氧化斷裂的情形，以及氧化代謝產物 8-OHdG。結果顯示：四種葉綠素衍生物對 10 μM 的過氧化氫誘導淋巴球細胞氧化傷害都具有保護的作用；但是在 50 μM 的過氧化氫的誘導下，脫鎂脫植醇葉綠素 a 與 b 對淋巴球細胞氧化傷害仍有保護的效果。第二部份，將小鼠肝臟細胞株分別暴露在四種葉綠素衍生物 5~50μM 共同培養後，再給與 5、10 ng/mL 黃麴毒素誘導肝細胞的 DNA 損傷。利用 ELISA 測量黃麴毒素與 DNA 結合的產物結果顯示：四種葉綠素衍生物對於黃麴毒素誘導肝細胞的 DNA 損傷都具有保護的作用，推測葉綠素的結構具有捕捉分子的能力，可螯合黃麴毒素進而減少黃麴毒素對肝細胞基因的損傷。此外，在排除分子捕捉能力的研究中發現，脫鎂脫植醇葉綠素 a 與 b 對於肝細胞 DNA 的損傷仍具有保護的效果，且可以調節肝細胞中麩胱甘肽轉硫酶的活性，進而增加肝臟中對黃麴毒素的解毒功能。

Abstract

Chlorophylls (Chls) are the most abundant natural plant pigments. Four chlorophyll derivatives, including chlorophyllide a and b (Chlide a and b) and pheophorbide a and b (Pho a and b), were investigated for their in vitro antioxidative capacities and anticytotoxicity properties to the cell DNA damage. First, the antioxidative effects of four chlorophyll derivatives on hydrogen peroxide (H_2O_2)-induced strand breaks and oxidative damage were evaluated in human lymphocyte. Lymphocytes exposed to H_2O_2 at a concentration of 10 and 50μM revealed an increased frequency of DNA single-strand breaks (ssbs; as measured by the comet assay) and also the level of oxidized nucleoside (as measured by 8-hydroxy-2-deoxyguanosine; 8-OHdG). All Chls reduced the level of DNA ssbs and 8-OHdG within human lymphocytes following exposure to 10μM H_2O_2. Only Pho a and b were able to decrease DNA ssbs and 8-OHdG following treatment of lymphocytes with 50μM H_2O_2, in a concentration-dependent fashion. It was demonstrated herein that Pho a and b, were more antioxidative than others. We applied DPPH free-radical scavenge assays *in vitro,* and also got similar results. Pho a and b were higher ability on scavenging capacities than others. In the second part, the inhibitory effects of four chlorophyll derivatives on aflatoxin B_1 (AFB_1)-DNA adduct formation, and on the modulation of hepatic glutathione S-transferase (GST) were evaluated in murine

hepatoma (Hepa-1) cells. Enzyme-linked immunosorbent assay (ELISA) showed that pretreatment with Chlide or Pho significantly reduced the formation of AFB_1-DNA adducts, and that Pho was the most potent inhibitor. However, wash-out prior to adding AFB_1 totally eliminated inhibition by Childe and partially eliminated inhibition by Pho, indicating that the inhibitory effect of Chlide, and to some extent Pho, was mediated through direct trapping of AFB_1. Furthermore, spectrophotometric analysis showed that Pho treatment could increase GST activity in Hepa-1 cells. These observations indicate that the chlorophyll derivatives studied may attenuate AFB_1-induced DNA damage in the Hepa-1 cell by direct trapping of AFB_1. Pho provided additional protection not only by direct trapping, but also by increasing GST activity against hepatic AFB_1 metabolites.

We conclude that water extract Chls are able to enhance cells' ability to resist H_2O_2-induced oxidative damage and (AFB_1)-DNA adduct formation, especially for Pho a and b.

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目錄

中文摘要……………………………………………………………I
英文摘要……………………………………………………………II
致謝……………………………………………………………IV
目錄……………………………………………………………VI
表目次……………………………………………………………X
圖目次……………………………………………………………XI
縮寫……………………………………………………………XIV
第一章　緒論……………………………………………………………1
第二章　文獻回顧……………………………………………………………4
第一節　氧化壓力與DNA損傷……………………………………………4
一、氧化壓力的產……………………………………………4
二、DNA損傷……………………………………………7
三、抗氧化防禦系統的保護機制……………………………………………8
第二節　黃麴毒素與DNA損傷……………………………………………11
一、黃麴毒素的代謝途徑……………………………………………11
二、黃麴毒素對於致腫瘤的影響……………………………………………13
三、黃麴毒素代謝物對細胞毒性的相關研究……………………………………………15
四、黃麴毒素導致DNA損傷的實驗研究模式……………………………………………17

第三節　葉綠素的性質與生理效應……….……..………………………...19

一、葉綠素的簡介……………...…………………………………………19

二、葉綠素的消化吸收…………………………………….………………26

三、葉綠素的保健功效在生物體內、體外的研究模式…………26

第三章　葉綠素的水萃取物質對過氧化氫所誘導淋巴球細胞 DNA 損傷之影響…………….……………………………..…………...32

第一節　研究方法…………………………………………………..……32

一、實驗材料…………………………………………………………32

二、分析項目與測量方法……………………………………………33

三、統計分析方法……………………………………………………46

第二節　結果………………………………………………………………47

一、葉綠素水萃取物純度分析………………………………………47

二、細胞存活率分析…………………………………………………51

三、清除 1,1-diphenyl-2-picryl-hydrazyl (DPPH)自由基能力..….54

四、H_2O_2 誘導 DNA 氧化損傷………………………………..……57

五、葉綠素對於 H_2O_2 誘導淋巴球細胞 DNA 氧化損傷的影響…60

第三節　討論………..…………………………………………………...65

一、植物性化學物質抗氧化性的比較..65

二、葉綠素在體內的代謝..65

三、葉綠素含量對人體淋巴球細胞的影響......................................66

四、銅鈉葉綠素製品的抗氧化作用..67

五、吡硃類(porphyrins)的抗氧化作用...69

六、天然葉綠素代謝產物的抗氧化比較..70

第四章　葉綠素的水萃取衍生物對黃麴毒素所誘導肝細胞 DNA 損傷之影響......72

第一節　研究方法......72

一、實驗材料......72

二、分析項目與測量方法......72

三、統計分析方法......81

第二節　結果..82

一、細胞存活率分析......82

二、黃麴毒素 B_1 誘導 DNA 損傷...84

三、葉綠素水萃取物對於 Hepa-1 細胞株 AFB_1-DNA adducts 產生的影響...86

四、葉綠素水萃取物對於 Hepa-1 細胞株 GST 活性的影響..........92

第三節 討論......96

一、植物性化學物質對抗黃麴毒素的探討..96

二、葉綠素對黃麴毒素誘導基因突變的保護探討....................................99

第五章　結論……………………………………………….……………..103

第六章　參考文獻…………………………..…………………………..104

第七章　已發表之期刊論文...125

表目次

表一、黃麴毒素致肝腫瘤的動物研究……...…………….……………....16

表二、黃麴毒素致肝腫瘤的人類流行病學研究……...………..…………16

表三、人類淋巴球細胞加入葉綠素水萃取物與過氧化氫培養後的存活率…....……………………………………………….........………. 52

表四、人類淋巴球細胞加入 H_2O_2 培養後的存活率………..……..……...53

表五、達到 50％清除 DPPH 自由基(IC50)所需要的葉綠素濃度.............56

表六、H_2O_2 對於人類淋巴球細胞氧化損傷的影響……………….…….58

表七、Hepa-1 細胞株對葉綠素水萃取物與黃麴毒素 B_1 培養後的存活率 83

表八、AFB_1 對於 Hepa-1 細胞株 DNA 損傷的影響………………………85

表九、AFB_1 對於 Hepa-1 細胞株 GST 活性的影響……………….....…93

圖目次

圖一、超氧歧化酶(SOD)的作用……..………………………………………6

圖二、Fenton 化學反應..…………………………………………………..6

圖三、黃麴毒素的化學結構………………..……………..……..………12

圖四、黃麴毒素 B_1-2,3-epoxide…………………………………………12

圖五、AFB_1 在體內的代謝途徑..………………………………………..…14

圖六、DNA 與黃麴毒素 B_1 結合的作用機制………………..…………..…18

圖七、Porphyrin 的化學結構………………..……………………..………22

圖八、Chlorophyll a 的化學結構……………………………………..………22

圖九、Chlorophyll b 的化學結構……………………………………..………22

圖十、葉綠素 a 的相關代謝物…………………………..……………………23

圖十一、葉綠素 b 的相關代謝物…………………………..………………24

圖十二、Chlorophyllin 的化學結構……………………….……..……………25

圖十三、Protoporphyrin 的化學結構…………………………..………………31

圖十四、MTS tetrazolium 與其 formazan 產物的結構式.......………..……......35

圖十五、在螢光顯微鏡下所觀察到 DNA 未損傷，以及損傷的情形……39

圖十六、彗星分析的流程圖……………………………………………...42

圖十七、葉綠素水萃取物-Chlorophyllide a 成分之 RP-HPLC 分析圖…47

圖十八、葉綠素水萃取物-Chlorophyllide b 成分之 RP-HPLC 分析圖…48

圖十九、葉綠素水萃取物-Pheophorbide a 成分之 RP-HPLC 分析圖……49

圖二十、葉綠素水萃取物- Pheophorbide b 成分之 RP-HPLC 分析圖……50

圖二十一、葉綠素水萃取物清除 DPPH 自由基的能力……………………55

圖二十二、以彗星分析 DNA 損傷的結果…………………………………59

圖二十三、添加各種不同濃度的葉綠素水萃取物質後再給與 10μM H_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(彗星分析)……………………………………………………………61

圖二十四、添加各種不同濃度的葉綠素水萃取物質後再給與 10μM H_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(8-OHdG)……………………………………………………62

圖二十五、添加各種不同濃度的葉綠素水萃取物質後再給與 50μH_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(彗星分析)……………………………………………………………...63

圖二十六、添加各種不同濃度的葉綠素水萃取物質後再給與 10μH_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(8-OHdG)……………………………………………………64

圖二十七、AFB_1-DNA adducts 標準曲線…………………………...…79

圖二十八、GSH 與 CDNB 的反應…………………………………….…81

圖二十九、Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後再

給與 5 ng/mL AFB_1 所誘導 DNA 損傷的結果…………………88

圖三十、Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後再給與 10 ng/mL AFB_1 所誘導 DNA 損傷的結果……………………89

圖三十一、Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後 wash out 再給與 5 ng/mL AFB_1 所誘導 DNA 損傷的結果……………………………………………………………………90

圖三十二、Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後 wash out 再給與 10 ng/mL AFB_1 所誘導 DNA 損傷的結果……91

圖三十三、Hepa-1 細胞株在給與 5 ng/mL $AFB_1$$AFB_1$ 前先加葉綠素水萃取物細胞內 GST 活性的結果……………………………………94

圖三十四、Hepa-1 細胞株在給與 10 ng/mL $AFB_1$$AFB_1$ 前先加葉綠素水萃取物細胞內 GST 活性的結果……………………………………95

圖三十五、Oltipraz 的化學結構…………………………...……………...…99

縮寫

8-OHdG	8-hydroxy-2-deoxyguanosine
AF	Aflatoxin
AFB_1	Aflatoxin B_1
ATP	Adenosine Triphosphate
Chls	Chlolophylls
Chlide a	Chlorophyllide a
Chlide b	Chlorophyllide b
Chlin	Chlorophyllin
CYP450s	Cytochrome 450s
DMBA	7,12-dimethylbenz [a]anthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPPH	1,1-diphenyl-2-picryl-hydrazyl
ELISA	Enzyme-Linked Immunosorbent Assay
ESR	Electron Spin Resonance
GC-MS	Gas chromatography-mass spectrometry
GSH	Glutathione
GST	Glutathione-S- transferase
H_2O_2	Hydrogen peroxide
Hepa-1	Hepatoma cell (Hepa-1c1c7)

I3C	Indole-3-carbinol
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NQO1	NAD(P)H:Quinone Oxidoreductase 1
PBS	Phosphate buffer saline
Pho a	Pheophorbide a
Pho b	Pheophorbide b
RNA	Ribonucleic Acid
RP HPLC	Reserved Phase High Performance Liquid Chromatography
ROS	Reactive oxygen species
SOD	Superoxidase dismutase
Ssbs	Single-strand breaks
SCGE	Single cell gel electrophoresis
TBARS	Thiobarbituric acid reactiove substance

第一章　緒論

癌細胞的形成，是先天的基因因數與後天暴露的環境因數交互作用的結果，且常與細胞的遺傳物質-去氧核醣核酸(deoxyribonucleic acid；DNA)損傷有關。其中環境誘發 DNA 損傷的原因頗多，包括：紫外線、游離輻射的暴露、活性氧物質(reactive oxygen species；ROS)產生自由基的攻擊，或是化學性致癌物質的接觸等等，一旦 DNA 遭到損傷，在修補過程中，可能會因為無法正常的修復而產生突變，導致遺傳物質累積錯誤而使細胞癌化。

不良的飲食容易導致細胞 DNA 損傷，例如油炸或醃製、碳烤食品(例如香腸、熱狗、烤肉等等)中所產生的過氧化物質或多環芳烴類，以及食物中潛在的化學性致癌前驅物質，包括食品本身如黃樟素，或因為不當貯存時所產生的毒素如黃麴毒素等。長期暴露於上述的飲食中，體內 DNA 容易被破壞而產生突變，進而增加罹患癌症的風險。

世界衛生組織建議每日蔬果適當攝取量為 400 至 800 公克；因此自一九九一年起，世界相關健康團體或防癌組織即推動全球性飲食防癌運動，稱為「5 a Day for Better Health（一日五蔬果好健康）」，鼓勵民眾每天至少攝取五份新鮮的蔬菜水果。近年來根據流行病學的調查研究顯示：多攝取蔬菜水果可以顯著的減少各種癌症的罹患率，亦即蔬果具有防癌的效果。一般認為是與蔬果中所含的植物性化學物質

(phytochemicals)有關，葉綠素(chlorophylls；Chls) 便是其中之一。

葉綠素是植物中含量最豐富的植物色素之一，其中深綠色的蔬菜是飲食中葉綠素的主要攝取來源。法國化學家 Pelletier 與 Caventou 在 1818 年將植物葉片中所見到的綠色物質命名為 Chlorophyll，即葉綠素。葉綠素主要的功能是吸收光能，並藉由電子傳遞鏈將能量貯存於三磷酸腺苷(adenosine triphosphate；ATP)中，在植物進行光合作用的過程中扮演著不可或缺的角色。自然界中葉綠素超過 50 個成員，由 Tetrapyrrolic ring 形成分子的架構，再由環中央的鎂(Mg^{2+})離子以及植醇 (phytol)所構成。然而蔬果中的葉綠素常常隨著生長、凋零、烹調時的加工或加熱，使得這些葉綠素分解成許多衍生物，其中脫去植醇的葉綠素為水溶性，常見於蔬菜的湯汁、果汁以及茶飲料中。無論是台灣民間傳統標榜有清肝、解毒功能的青草茶(Chinese herb tea)，或是西洋國家宣稱有養生美容效果的小麥草汁(wheatgrass juice)，甚至日、韓近年流行以健康為主要訴求的大麥若葉エキス-青汁以及蔬菜湯都富含水溶性的葉綠素。

早期葉綠素對人體保健功效的研究僅發現它具有抑菌、除臭的功能。而各種天然葉綠素衍生物質在抗氧化及抗基因突變的研究文獻並不多見，近年來則以葉綠素的人工合成的水溶性製劑-銅鈉葉綠素(chlorophyllin；Chlin)為研究的對象，然而它並非存在於自然的食物中。本研究是第一個將食物中的各種水溶性葉綠素萃取後，對活體細胞或是

細胞株進行抗氧化或抗基因突變的實驗，目的是希望藉由營養科學的研究方法來探討天然葉綠素對生物體的保健功效，進而證實每日從飲食中攝取綠色蔬果的重要性。

第二章　文獻回顧

第一節　氧化壓力與DNA損傷

一、氧化壓力的產生

氧氣對我們的生活非常重要，人體的每個細胞，皆需要氧氣來進行氧化作用。氧化作用的過程可以將能量營養素(例如醣類、脂肪或蛋白質)分解而釋出能量，人體便藉由此能量的獲得而賴以生存。然而，人體細胞在進行氧化作用的同時也會產生自由基(free radicals)對細胞造成氧化傷害。所謂的自由基是指構成物質的原子團在電子軌域上具有不成對電子的情形，這種電子結構極不穩定，易與其他分子發生反應。在所有自由基種類中，以氧為中心的自由基與其代謝產物稱為活性氧分子，包括氫氧自由基(hydroxyl radical, $OH^{\cdot}$)，超氧陰離子(superoxide anion, $O_2^{\cdot-}$)，單重態氧(singlet oxygen, $^1O_2$)以及過氧化氫(hydrogen peroxide, H_2O_2)等，對人體健康的危害最大。由於ROS大多都不穩定，因此極易與其他分子碰撞而形成許多連鎖反應(chain reaction)，進而產生更多的ROS。ROS的生成有以下兩個來源

(一)、生物體內正常代謝產生

ROS主要生成於細胞內的粒線體、內質網、細胞膜、核膜等(Gutteridge and Halliwell, 1990)。目前已知生物體內產生ROS的來源包括：

1.自發性氧化還原反應(auto-oxidation)：例如核黃素(flavin)的自我氧化作用會產生$O_2^{-\cdot}$(Massey, 1994)。

2.電子傳遞鏈反應(electron transfer chain)：生物體在正常的呼吸作用下，O_2自粒線體電子傳遞鏈獲得電子後，便會產生$O_2^{-\cdot}$，$O_2^{-\cdot}$在超氧歧化酶(Superoxide dismutase；SOD)的作用下產生H_2O_2(圖一)。此外H_2O_2在二價鐵的存在下進行Fenton作用，產生更具毒性的$OH^{\cdot}$(圖二)。

3.代謝反應過程中產生的中間產物：例如黃嘌呤氧化酶(xanthine oxidase)將黃嘌呤代謝成尿酸(uric acid)，將伴隨產生H_2O_2與$O_2^{-\cdot}$(Janero, 1990)。

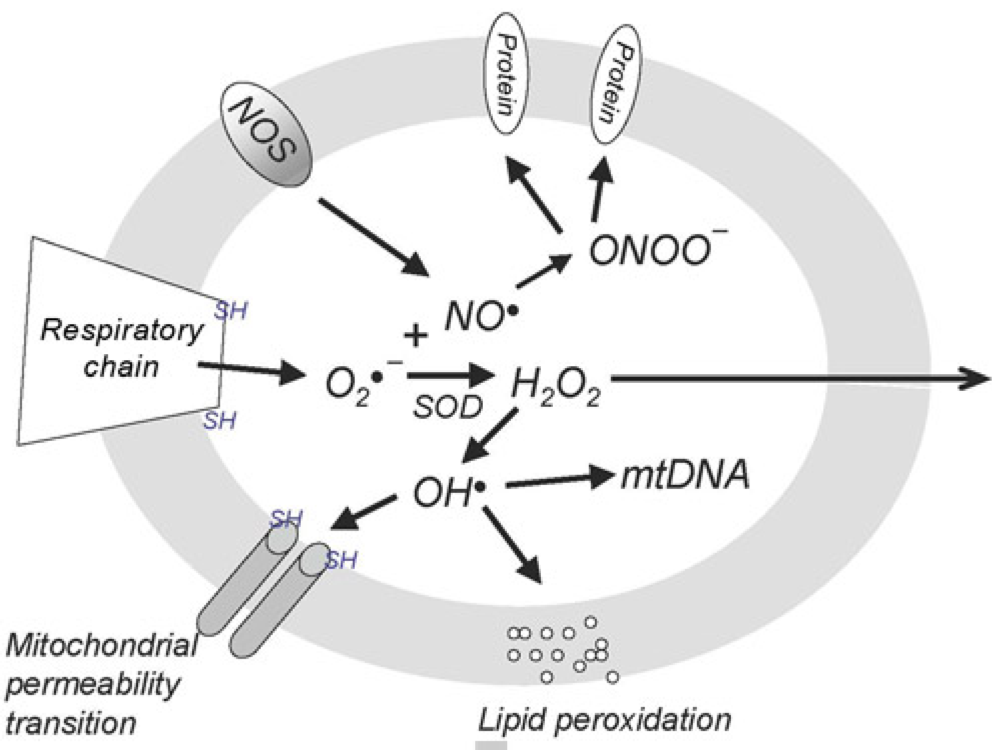
4.白血球的發炎免疫反應：白血球在吞噬外來物質時，會產生O_2^{-}來弒殺外來物質以對抗外來物質的侵襲(Nishikawa, et al., 1999)。

(二)、外在環境暴露

環境中的ROS主要來源包括以下：

1.輻射、紫外線以及電磁波：日光曝露或癌症患者接受放射線治療都會產生自由基，因為生物體持續暴露在遊離輻射下，會使體內的水分子裂解而產生$OH^{\cdot}$(Hagen, 1989) 。

2.環境污染：交通運輸工具所排放出來的燃油飛灰(oil fly ash)，伴隨產生許多ROS。環境污染包括空氣、飲用水以及土壤等(Kadiiska, et al., 1997)。



圖一：超氧歧化酶(SOD)的作用

Figure 1：The reaction of SOD (Szeto,2006)

$$Fe^{2+} + H_2O_2 \dashrightarrow Fe^{3+} + {}^{\cdot}OH + OH^{-}$$

圖二：Fenton化學反應

Figure 2：Fenton reaction

Glossary of terms used in bioinorganic chemistry (IUPAC Recommen dations 1997) on page 1274

3.毒性化學物質暴露：例如不當的食品添加劑(例如漂白劑中含有H_2O_2)、農藥、被毒素污染的食品(例如黃麴毒素)以及毒品等(Jayashree and Subramanyam, 2000)。
4.精神壓力狀況：現代都市人，壓力過大、急躁、焦慮、鬱悶、緊張等情緒問題，也會產生ROS (Hapuarachchi, et al., 2003)。

一旦體內ROS的產量超出人體天然防禦的範圍，可和體內許多重要分子如核酸、蛋白質、或生物膜上之多元性不飽和脂肪酸反應，形成氧化壓力 (oxidative stress)，導致生物體氧化性傷害。主要之反應為自由基，容易引發細胞膜上之不飽和脂肪酸進行脂質過氧化反應之外，並會與膜上酵素或接受體行共價結合，破壞細胞膜的完整性，改變其結構功能及通透性；另外自由基亦可和細胞內之蛋白質行交錯連結反應致使蛋白質變性或結構改變，喪失酵素活性，進而使細胞內之正常功能無法進行；同時自由基亦會攻擊DNA分子，破壞其鹼基結構使其功能改變，造成基因突變。

二、DNA損傷

許多因素會造成 DNA 的傷害，其中大致區分成自然突變以及外在突變兩大項。自然突變發生在負責複製 DNA 的酵素，如 DNA 聚合酵素(DNA polymerase)在複製的過程中將錯誤的鹼基引入 DNA 中，而產生 DNA 的配對錯誤。而外在的突變包括細胞受到外界的

遊離輻射、紫外線或化學物質的破壞。其中自由基及活性氧的產生，乃因其與體內的抗氧化抵禦系統未能達平衡時所產生的氧化壓力，此氧化壓力產生可能導致體內抗氧化防禦系統的耗盡、脂質的過氧化、細胞膜的損壞、DNA 的破壞。與自由基的傷害有關的疾病包括：動脈粥狀硬化(atherosclerosis)、氣腫(emphysema)、潰瘍性結腸炎(ulcerative colitis)、糖尿病(diabetes)、風濕性關節炎(rheumatoid arthritis)、巴金森氏症(Parkinson's disease)及癌症(cancer)等(Machlin and Bendich, 1987；Briges, et al., 1992； Bonorden and Pariza, 1994) 。

許多的研究顯示氧化壓力可導致 DNA 損傷，包括 DNA 片斷化和程式凋亡(Dreher and Junod, 1996)、鹼基修飾(Kasai, et al., 1984)及 DNA 單/雙股的斷裂(Sarker, et al., 1995)。DNA 的氧化修飾被認為與致突變性(mutagenesis)和致癌性(carcinogenesis) (Dreher and Junod, 1996; Okamoto, et al., 1996)有關。因此，Halliwell 在 2000 年指出 DNA 氧化損傷可作為罹患癌症的風險生物指標(biomarker)，因此若是能夠預防或降低 DNA 損傷，就可避免正常細胞發生癌化的作用(carcinogenesis)。

三、抗氧化防禦系統的保護機制

流行病學的調查研究顯示：多攝取蔬菜水果可以顯著的減少各

種癌症的罹患率，亦即蔬果具有防癌的效果（Block, et al., 1992）。而攝取蔬菜水果被認為具有降低慢性疾病發生的原因，一般認為是與蔬果中所含的抗氧化物質有關(Foyer and Fletcher, 2001)，包括：類胡蘿蔔素、維生素 C、維生素 E、硒（Young and Lowe, 2001; McDermott, 2000）、類黃酮（Slobodan and Michael, 2000）、多酚類（Wolf and Christa, 2002）等。

體內的抗氧化系統包括抗氧化酵素，諸如：超氧岐化酶(superoxide dismutase；SOD)、觸酶(catalase)、麩胱甘肽過氧化酶(glutathione peroxidase；GSHPx)、麩胱甘肽轉移酶(glutathione -S-transferase；GST)等，以及抗氧化劑諸如：維生素 E、維生素 C、胡蘿蔔素、GSH、尿酸(urate)、Carnosine 及 Anserine 等，甚至一些可與過渡元素金屬結合之化合物，諸如：白蛋白(albumin)、轉鐵蛋白(transferrin)及藍胞漿素(ceruloplasmin)亦具有抗氧化能力(Bonorden and Pariza, 1994)。自然界亦存在一些非營養價值之抗氧化物質，諸如：酚類(phenolic compounds)、Furanones、單寧酸(tannins)及 Phenylpropenoids 等。這些抗氧化物質在生物體內的作用原理包括：自由基終止劑（如：維生素 E)、還原劑或清除劑（如：維生素 C）及單旋態氧的抑制劑（如：胡蘿蔔素）(拱玉郎, 1997)。此外，番茄紅素(lycopene)、花青素（anthocyanin）及兒茶素（catechin）等

（錢明賽 1998），因為具有苯環及雙鍵結構的多酚類，因此可以發

揮其抗氧化的特性。

第二節　黃麴毒素與DNA損傷

黃麴毒素(aflatoxin；AF)是環境中重要的致癌物質之一，由 *Aspergillus flavus* 及 *Aspergillus parasiticus* 代謝所產生的次級代謝產物。依其在紫外光下之螢光性可分為藍色螢光的 B_1、B_2，以及綠色螢光的 G_1、G_2 (Schuller, 1976)如圖三。這四種黃麴毒素中結構相似，含有夫喃環(furan ring)以及內酯環(lactone ring)的結構，其中以 B_1 存在最廣，且毒性最強。

一、黃麴毒素的代謝途徑

黃麴毒素 B_1(aflatoxin B_1；AFB_1)的代謝主要在肝臟，經過 Phase I 酵素，即肝臟內的細胞色素 P450 酵素系統(cytochrome 450s；CYP450s)酵素的代謝，雖然此步驟是動物體保護抵抗外來侵犯物質的防禦系統之一，但卻常使得 AFB_1 氧化成具有高度親電子性(electrophilic)的不穩定環狀氧結構-AFB_1-epoxide derivatives(圖四)，成為致癌的活性物質。例如在老鼠的實驗中，以 Phenobarbital 誘導 CYP 2B1 活性，會促使 AFB_1-epoxide 衍生物增加(Gurtoo, 1980)；CYP 3A4 (nifedipine oxidase)被認為是人類體內將 AFB_1 活化為 AFB_1-2,3-epoxide 的最主要酵素(Shimada and Guengerich, 1989)。另一方面，部分的 CYP450 酵素系統亦具有降低 AFB_1 的毒性；例如 Koser 等學者(1988)的研究發現，於 C57BL/6J 雌性小鼠腹腔注射

Aflatoxin B1　　Aflatoxin B2　　Aflatoxin G1　　Aflatoxin G2

圖三：黃麴毒素的化學結構

Figure 3: The structure of aflatoxins

圖四:黃麴毒素 B_1-epoxide 的產生

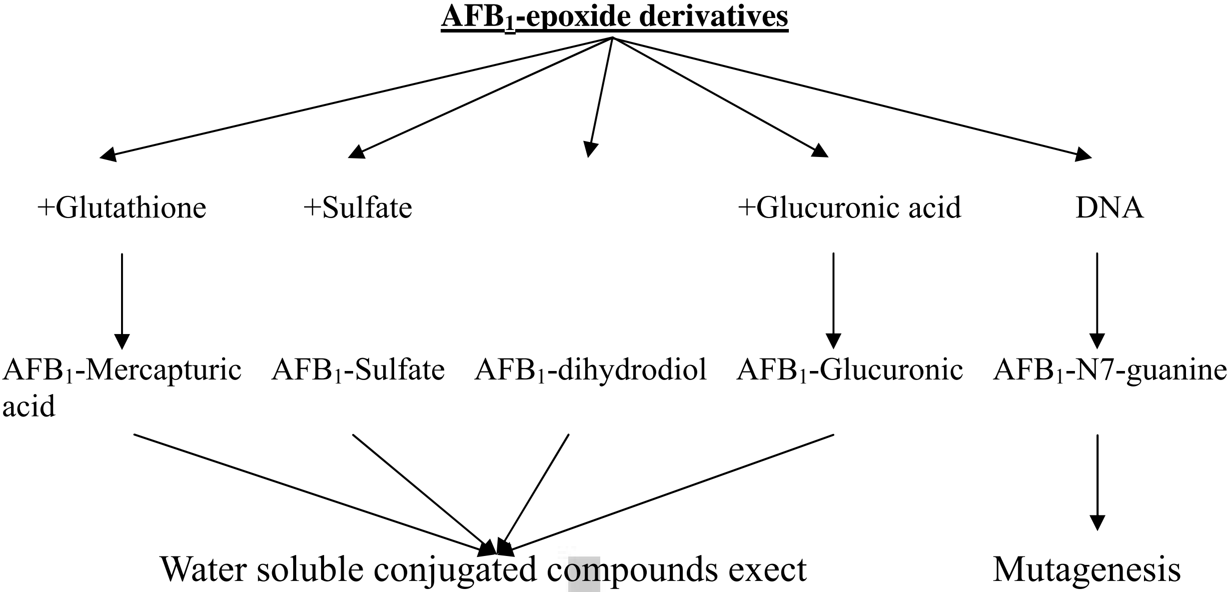
Figure 4:　Form reactive aflatoxin B_1-epoxide

β-Naphthoflavone 誘導 CYP 1A2(AFB_1-4-hydroxylase)活性，會促使 AFB_1 代謝成較低毒性的代謝產物-4-OH-AFB_1，因而降低 AFB_1 的毒性。

圖五表示 AFB_1-epoxide 衍生物在體內繼續的代謝途徑。一般而言，大部分被活化的 AFB_1-epoxide 會經由細胞內的 Phase II 酵素，即麩胱甘肽轉移酶(glutathione-S-transferase；GST)的催化作用，與麩胱甘肽(glutathione；GSH)結合而形成親水性較高的產物，再經由膽汁排出體外達到解毒的目的(Prochasks, et al., 1985）。目前已知 GSTs 有許多 Isoform，與 AFB_1 結合有關的 GST isoform 在老鼠是屬於 GST-α (Coles, et al., 1985)；在人則是屬於 GST-μ (Janeric, et al., 1990)。

二、黃麴毒素對於致肝腫瘤的影響

首次被報導的黃麴毒素污染事件是在 1960 年於英國東南部，共有十萬隻火雞大量死亡，這些死亡的家禽都是餵食巴西一家飼料公司所生產的花生飼料；隔年證實這些飼料被黃麴黴菌(*Aspergillus flavus*)所分泌的毒素-黃麴毒素所污染(Sargeant, et al., 1961)。獸醫師解剖中毒死亡的家禽發現普遍有肝壞死(hepatic necrosis)、膽管增生(bile duct proliferation)以及肝基質退化(parenchymal degeneration)的現象。1963 年，Butler 與 Barnes(1963)以含有定量 AFB_1 的花生餵食老鼠，證實會誘發肝癌。



圖五: AFB_1-epoxide 在體內的代謝途徑

Figure 5: The metabolic pathway of AFB_1-epoxide

動物實驗已證實 AFB_1 可引起鱒魚(trout)、鴨子(duck)、大白鼠(rat)以及恆河猴(rhesus)等動物的肝腫瘤(表一)。由於種系之差別，各種動物對 AFB_1 的敏感性亦有差異，例如小鼠(mouse)以及中國倉鼠(hamster)對 AFB_1 的抗性較強，除了部分品系外，其他不易發生肝腫瘤現象。

根據流行病學調查的結果，AFB_1 可能是造成亞洲與非洲地區居民肝癌發生率偏高的原因之一。許多研究者調查居民的尿液、食物以及農產品中黃麴毒素的含量，發現黃麴毒素與肝癌有極高的相關性(表二)。因此我國衛生署食品衛生標準規定，花生及其製品中黃麴毒素不得超過 15ppb。

三、黃麴毒素代謝物對細胞毒性的相關研究

AFB_1 的活化是引起毒性效應最重要的一環。AFB_1 本身並不能直接導致癌症的發生。由圖五可知 AFB_1-2,3-epoxide 可以藉由體內酵素系統代謝而排出，但 AFB1-2,3-epoxide 也可進一步與細胞內的 DNA、核糖核酸(ribonucleic acid, RNA)或是蛋白質等大分子物質結合形成鍵結產物(adducts)，對細胞產生致突變性的作用(Swinson, et al., 1977)。由於 AFB_1-2,3-epoxide 是在碳與碳間介入一個氧原子，為一個非常不穩定的結構，會與 DNA 中的 Guanine 形成共價鍵結(圖六)，

表一：黃麴毒素致肝腫瘤的動物研究

Table 1: Aflatoxin induced hepatoma in the animal model

種別	年齡	性別	實驗方法	觀察期間	產生肝腫瘤百分比	作者
鱒魚(trout)	小魚	M & F	飲食中 8 μg/Kg	16 月	80%	Busby, et al.
鴨子(duck)	7 天	M & F	飲食中 30 μg/Kg	14 月	73%	Carnaghan
大白鼠(rat)	28 天	M & F	飲食中 1.0 mg/Kg	41-64 週	86%	Wogan,et al.
恆河猴(rhesus)	9 天	M & F	注射 99-1354 mg	39-147 月	62%	Sieber, et al.

表二：黃麴毒素致肝腫瘤的人類流行病學研究

Table 2: Aflatoxin induced hepatoma in the human research

地點	研究方法	研究結果	作者
非洲莫三比克 Mozambique	蒐集 1965-1975 年癌症登記的資料，並採集居民食物樣本分析 AF 含量。	肝細胞癌的發生和 AF 攝食量的對數值成顯著的相關。	Rensburg, et al.
非洲史瓦濟蘭 Swaziland	將全國分成十個小區域，蒐集 1979-1983 年肝癌的發生率、B 型肝炎病毒帶原及食物、農產品中 AF 的含量。	AFB_1 對於肝癌的發生率的相關性比 B 型肝炎的相關性高。	Peers
非洲肯亞 Kenya	收集部份地區之 1978-1982 年肝癌的發生率，以抽樣採集居民血液，分析尿中 AFB1-N7 Gua 含量以及 B 型肝炎帶原的情況。	AFB_1 的暴露與肝癌的發生率有相關。	Autrup
亞洲台灣 Taiwan	以 134 位肝癌病人進行病例對照分析。	常吃發酵食品者，發生肝癌的危險性為不常吃的 4.7 倍，但是樣本數偏低。	Chen
亞洲中國 China	從 1982 年開始追蹤 25-64 歲居住在廣西地區的男性肝癌的死亡率情形，並分析居民食物樣本中 AFB1 的含量。	AFB_1 暴露量與肝癌死亡率有很強的線性關係。	Yeh

最常發生的位置是在 Guanine N-7，導致造成 DNA 上的鹼基發生 G:C →T:A 突變(Forster, et al., 1983)。

AFB_1 除與 DNA 結合造成細胞突變毒性外，氧化傷害也是 AFB_1 造成 DNA 損傷，進而致腫瘤發生的原因之一(Shen, et al., 1996)。1995 年 Shen 等學者利用大白鼠腹腔注射單一劑量(100 μg/100 g wt)的 AFB_1 後，發現可誘發脂質過氧化(lipid peroxidation)以及 8-氫氧 2'-去氧鳥糞核糖核苷(8-hydroxy-2- deoxy- guanosine；8-OHdG)的產生，證實 AFB_1 會引起肝細胞的氧化性傷害及 DNA 單股斷裂的情形。若事先投予抗氧化劑(如維生素 E、硒)，發現維生素 E 可抑制 AFB_1 所引起的脂質過氧化的現象；硒可抑制脂質過氧化及 8-OHdG 的產生，因此推測 AFB_1 所引發的氧化性傷害是藉由 ROS 所產生。

四、黃麴毒素導致DNA損傷的實驗研究模式

利用競爭性酵素免疫分析法(competitive ELISA)，其原理是利用兩種抗原(一為固定量的標準品，一為待測樣品)共同競爭一定量的抗體，以此來定量待測樣品中的抗原量。本實驗與長庚大學謝玲玲教授合作，利用謝教授所研發成功的「抗 Iro-AFB1-DNA adduct」單株抗體 6A10 來測量 Aflatoxin B_1-DNA adducts 的含量(Hsieh, 1993)。

Base adduct: aflatoxin

aflatoxin

⇓ liver activation

⇓ DNA incorporation

aflatoxin – DNA adduct

圖六：DNA 與黃麴毒素 B_1 結合的作用機制

Figure 6: The mechanism of aflatoxin-DNA adducts formation

第三節　葉綠素的性質與生理效應

一、葉綠素的簡介

早在十七世紀末，科學家們即發現葉綠素是參與光合作用的主要色素，它存在植物細胞內的葉綠體中，當綠色植物在日照的條件下，利用二氧化碳與水能使綠色植物進行光合作用而獲得碳水化合物。在光合作用的反應過程中，藉由葉綠素一系列的的化學反應，將日光輻射能轉為化學能。

葉綠素反射綠光並吸收紅光和藍光，使植物呈現綠色。近年葉綠素的重大研究，首推德國的化學家 Willstatter，他訂出植物色素和葉綠素的化學結構，發現在綠色植物細胞中存在兩種類型的葉綠素 a 及 b，且它們都是含鎂的化合物，在綠葉細胞中約以三比一的量存在，因此於 1915 年獲諾貝爾化學獎。隨後，Fischer 繼續從事有關膽紅素和葉綠素的性質及結構方面的研究，認為血液、膽汁、及植物葉之中的色素組成都是由 4 個 Pyrrole 所組成的 Porphyrins 結構，並於 1929 年以人工合成 Haemin，隔年亦成為諾貝爾化學獎得主。

目前已知葉綠素的化學結構有超過一百種的變異存在，一片含有七千萬個細胞的葉子，約擁有五十億個葉綠體(chloroplast)，其中每個葉綠體約含有六億個葉綠素分子。所有葉綠素分子都與類囊膜(thylakoid membrane)上某些特定的蛋白質結合為色素-蛋白複合

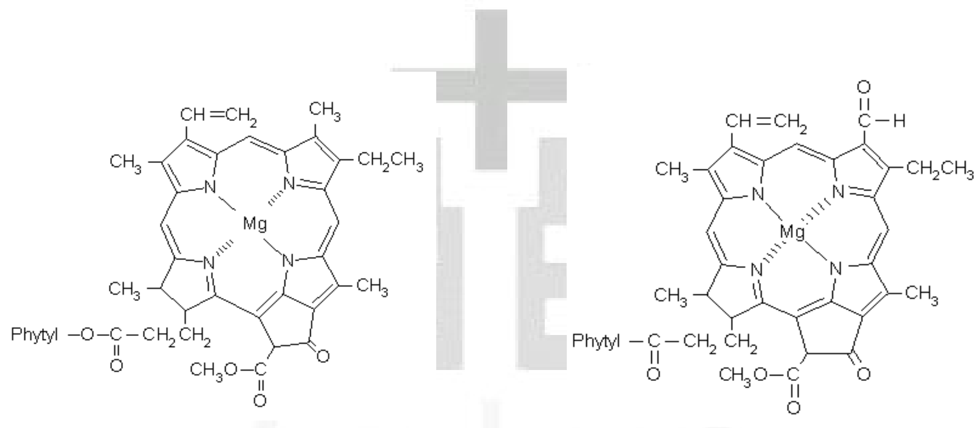
體，以提高捕捉日光和光合作用的效率。光合作用的的反應基本上區分為兩個階段，一為發生在類囊膜的光反應，二為發生於漿質的暗反應。前者是植物藉葉綠素吸收太陽光，把水分子裂解為氧分子、電子及氫離子，將其輻射能轉換為化學能 ATP，並透過 NADPH 累積還原力，此二光反應的產物即進入暗反應，協助漿質中循環的酵素系統以固定空氣中的 CO_2，使之轉換為葡萄糖。光反應所形成的 O_2，經過滲透及擴散後進入空氣中，提供地球上所有生物所需的 O_2。

一般而言，葉綠素的基本骨架結構主要由四個 Pyrrole 所組成，形成具有共軛雙鍵的巨環化合物，稱為吡啉(porphyrin)（圖七），其中四個氮原子螯合一個鎂離子，具有吸收光能的作用；另一端則為具有碳氫長鏈稱為植醇鏈 (phytol chain)，在生物體中此一長鏈可使葉綠素固定在一定的位置。一般而言，在高等植物中只有 chlorophyll a (Chl a)（圖八） 與 chlorophyll b (Chl b)（圖九）兩種，但是若將新鮮的植物體或葉綠素溶液，在不同的生長或環境控制下（例如高溫短時間），就會生成不同的代謝衍生物；以 chlorophyll a 為例，若化學結構上脫去尾鏈的植醇鏈則形成 chlorophyllide a (脫植醇葉綠素，Chlide a)；若失去中心的鎂離子則形成 pheophytin a (脫鎂葉綠素，Phe a)，若 pheophytin a 進一步的脫去植醇鏈則形成 pheophorbide a (脫鎂脫植醇葉綠素，Pho a)的結構（圖十、圖十一）。此外，市售

的葉綠素製品，利用化學合成的方式將鎂離子去除，改為中心含銅離子，且較具有水溶性的鈉鹽，稱為銅鈉葉綠素 chlorophyllin (Chlin)（圖十二）。

圖七：Porphyrin 的化學結構

Figure 7: The chemical structure of porphyrin

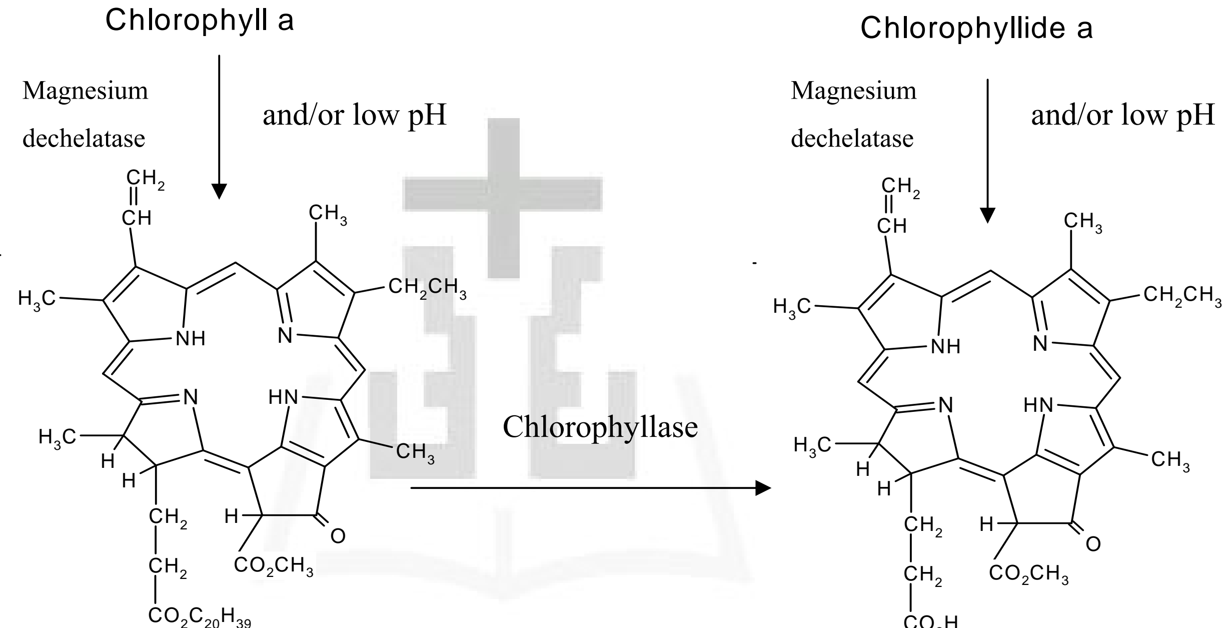


圖八：Chlorophyll a 的化學結構

Figure 8: The chemical structure of chlorophyll a

圖九：Chlorophyll b 的化學結構

Figure 9: The chemical structure of chlorophyll b



Chl a ＝893.5　　Phytol：C20H39＝279　　Chlide a ＝ Chl a – Phytol + 1H ＝893.5-279+1＝615.5

Phe a ＝ Chl a - Mg + 2H ＝893.5-23+2＝872.5　　Pho a ＝ Chl a - Mg + 2H – Phytol + 1H ＝893.5-23+2-279+1＝594.5

Chl a：Chlorophyll a,　Chlide a：Chlorophyllide a,　Phe a：Pheophytin a,　Pho a：Pheophorbide a

圖十:葉綠素 a 的相關代謝物

Figure 10: The related metabolites of chlorophyll a

Chlorophyll b → (Chlorophyllase) → Chlorophyllide b

Chlorophyll b → (Magnesium dechelatase and/or low pH) → Pheophytin b

Chlorophyllide b → (Magnesium dechelatase and/or low pH) → Pheophorbide b

Pheophytin b → (Chlorophyllase) → Pheophorbide b

Chl b =907.5　　Phytol：C20H39＝279

Chlide b ＝ Chl b – Phytol + 1H ＝907.5-279+1＝629.5　　Phe b ＝ Chl b - Mg + 2H ＝907.5-23+2＝886.5

Pho b ＝ Chl b - Mg + 2H – Phytol + 1H ＝907.5-23+2-279+1＝608.5

Chl b：Chlorophyll b,　Chlide b：Chlorophyllide b,　Phe b：Pheophytin b ,　Pho b：Pheophorbide b

圖十一:葉綠素 b 的相關衍生物

Figure 11: The related metabolites of chlorophyll b

圖十二 Chlorophyllin 的化學結構

Figure 12: The chemical structure of chlorophyllin

二、葉綠素的消化吸收

有關葉綠素在體內的消化吸收，早期的研究發現，利用 ^{14}C 標定的葉綠素-Phe a 給予正常人與罹患 Refsum 疾病的患者，顯示無論是正常人與罹患 Refsum 疾病的患者，其糞便中所排出的葉綠素代謝物量達 95%，顯示有 5%的葉綠素可被入體吸收(Baxter, 1968)。

從細胞的研究中亦發現，利用模擬人體小腸的 Caco-2 細胞模式，發現 Phe 可以經由體內的乳糜化作用，形成乳糜微粒，接著被小腸 Caco-2 細胞吸收，進入循環系統(Ferruzzi, et al., 2001)，因此間接證實葉綠素所產生的衍生物質，可以透過人體中的小腸黏膜細胞被吸收。在動物實驗的研究上，近年來中研院植物所楊棋明副研究員，利用哺乳類動物-紐西蘭白兔餵食新鮮菠菜 100 公克後發現，動物體內的各臟器與血液中都有葉綠素的代謝衍生物-Chlide a、b，Pho a、b 的形式出現(葉, 2003)。此外，海洋動物會經由食物攝取得到藻類或細菌葉綠素，其體內的 tetrapyrrole 結構皆為葉綠素的衍生物(Bortlik, et al., 1990)。以上的研究證實葉綠素所產生的衍生物，可以透過人體中小腸黏膜細胞被吸收，且葉綠素的環狀結構似乎可以被完整的保留，而發揮其抗氧化或抗突變性的效果。

三、葉綠素的保健功效在生物體內、體外的研究模式

關於葉綠素的應用，早在 1944 年 Smith 以葉綠素去處理表面傷

口，發現具有抑菌的功能。1951 年 Weingarten 與 Payson 發現，給予大腸造瘻的病人口服含銅鈉葉綠素後，可以消除身體所散發的異味，同時對於老年人常有的腸胃性症狀如便秘或腹脹的情形也頗有改善(Young and Bergei, 1980)。

近年來，則以葉綠素抗突變性的相關研究最多，Lai 在 1979 年的實驗中，將小麥芽的葉子水萃取物進行 ***Salmonella typhimurium assay***，發現它可以抑制 3-Methylcholanthrene 與 Benzo[a]pyrene 所引起的突變，且隨著蔬菜中葉綠素的含量愈多，其抑制的效果愈佳(Lai, et al., 1980)。銅鈉葉綠素(Chlin)也對於一些致癌物質如 N-methyl-N'-nitro-N- nitrosoguanidine (MNNG)、N-methyl-nitrosourea 有降低突變性的作用(Warner, et al., 1991)。此外，Chlin 對於環境或飲食中的致突變性的物質如香菸、烤焦的肉類或空氣中的煤塵等，具有競爭性的抑制 ***Salmonella typhimurium*** 產生突變的作用(Ong, et al., 1989)，如果與其他抗突變性的營養素(如 vitamin A、vitamin C、vitamin E、retinoic acid 以及 β-carotene)相比較，Chlin 抑制黃麴毒素所誘導的細胞突變效果最佳(Whong, et al., 1988；Ong, et al., 1989)。

葉綠素對抗突變的作用，同時也在動物的實驗中得到證實；例如果蠅幼蟲在給予綠藻或菠菜中萃取出的葉綠素或 Chlin 可以抑制 4-nitroquinoline 1-oxide、3-hydroxyamino-1- methyl-5H-pyrido [4,3-b]

indole 等具遺傳毒性的物質所造成的突變 (Negishi, et al., 1997)；哺乳類動物的實驗中，雄性老鼠在給予致突變物質-2-amino-3-mrthyl imidazo[4,5-f]quinoline (IQ)之前，先灌食 Chlin 會增加致突變物質(IQ)代謝產物的排出量(Dashwood and Liew, 1992)。以上無論是細胞層面或是活體動物的研究都發現，葉綠素或 Chlin 具有抗突變的作用。

Sato 等學者在 1984 年以水草進行抗突變實驗，發現含有 porphyrin 化學結構的葉綠素要具有抗致突變性必須是具水溶性、且對熱穩定的大分子。因此大部分研究所使用的葉綠素材料，常利用化學加工合成方式，將原來脂溶性的葉綠素 a、b 中的鎂離子，置換為含銅離子的銅鈉葉綠素(圖十二)，以獲得較具水溶性且安定的結構。但若回歸到人類飲食型態的角度而言，分析人類所攝取的綠色蔬果，發現其中的葉綠素會隨著植物生長、採收貯存或烹調而產生變化，分解成其他的葉綠素衍生物(圖十、十一)，其中以 Chlide a、b 及 Pho a、b 四種葉綠素的衍生物因其結構脫去植醇鏈，而成為較具水溶性，且對熱安定的化合物，推測亦應具有較佳的抗突變性效果。因此若能證實這些天然的葉綠素代謝衍生物具有抗細胞毒性的作用，將能提供攝取綠色蔬果有益健康的直接證據。

葉綠素除具有抗突變的作用外，亦具有抗氧化的作用；研究指出：葉綠素具有抗氧化的功能，因而可以抑制肝臟脂質的過氧化作

用(Sato, et al., 1977)。老鼠肝細胞中的粒線體在受到各種不同的活性氧或自由基的作用下，Chlin 可以有效的保護粒線體 DNA 不受到氧化傷害(Kamat, et al., 2000)。若從葉綠素的分子結構來分析，由於葉綠素屬於 porphyrin 類的物質，含金屬離子的 porphyrin 類物質可以抑制脂質的過氧化作用(Day, et al., 1999)。包括具有清除超氧自由基 O_2^-（Pasternack, et al., 1981）、 H_2O_2（Day, et al., 1997）與捕捉 peroxynitrite 的能力（Szabo, et al. 1996），其中，含有錳元素的 porphyrin 類物質，對於 H_2O_2 所誘導的肺纖維細胞粒線體 DNA 損傷，具有抗氧化保護的能力(Milano and Day, 1999)。此外，從電子自旋共振(electron spin resonance, ESR)的研究結果指出，Chlin 具有捕捉活性氧的能力(Kumar, et al., 2001)。

前面曾提及，肝臟的解毒酵素系統主要是以細胞色素 P450 為主的 Phase I 酵素，以及以 GST 為主的 Phase II 酵素。惟研究葉綠素對於肝臟解毒酵素的研究較少。過去研究植物性化學物質對於肝臟細胞解毒酵素的影響，包括含硫化合物(Teel and Huynh, 1998)、綠茶的萃取物(Hayashi, et al., 1992)、胡蘿蔔萃取物(Bishayee, et al., 1995)以及類黃酮素(Zuber, et al, 2002)等。葉綠素的 porphyrin 結構與細胞色素 P450 也有相關，Cobalt protoporphyrin(圖十三)會降低肝細胞中細胞色素 P450 的含量(Sinclair, et al., 1982）。此外，Yun 等學者 在 1995 年

以人類及老鼠的肝細胞微粒體(microsomes)為研究對象，探討葉綠素對 Phase I 酵素的影響，發現在以不同濃度的 Chlin 共同培養後，所測得的各種 P450 酵素皆明顯的受到抑制。因此推論葉綠素具有 porphyrin 的結構，對於 AFB_1 所引起肝毒性的保護作用，似乎有可能藉由抑制 P450 酵素的活性來達到降低 AFB_1 的活化。

對於 Phase II 酵素的影響，Lampe 等學者 (2000) 針對 57 位受試者給予三種不同的蔬菜飲食，為期各六天後發現，蔬菜的攝取，可以同時增加體內 GST-α 與 GST-μ 的活性，不過，由於蔬菜中的成分種類眾多，無法從此研究中推論出具體的有效成分為何？但是，既然是三種不同的蔬菜同時都具有活化 GST 的作用，那麼存在於各種蔬菜且含量居首的葉綠素是否調控重要的活化作用，值得深入探討。

圖十三：Protoporphyrin 的化學結構

Figure 13: The chemical structure of protoporphyrin

第三章　葉綠素的水萃取衍生物對過氧化氫所誘導淋巴球細胞 DNA 損傷之影響

第一節　研究方法

本研究的主旨為探討葉綠素及其衍生物對於過氧化氫所誘導的人類淋巴球細胞 DNA 損傷的影響。由於過去研究顯示，銅鈉葉綠素可以有效抑制 DNA 的氧化損傷，因此本研究以 Chlin 為對照實驗，與葉綠素的水萃取物作比較。

一、實驗材料

(一) 葉綠素水萃取物的製備

1.菠菜

葉綠素的水萃取物質是由中央研究院植物暨微生物學研究所楊棋明博士提供。新鮮的菠菜購自台灣農家。於購買後立即進行冷凍乾燥，並加入液態氮，以研缽磨成粉末狀後於-70℃下保存備用。

2.葉綠素萃取物的純化

首先利用膠體過濾法(CM-Sepharose CL-6B cation- exchange chromatography) 自菠菜中分離葉綠素 a、b。接著將 chlorophyllase 酵素分別加入葉綠素 a、b 中，靜置反應 30 分中後脫去尾鏈的植醇鏈而得到脫植醇葉綠素 a 與 b。將脫植醇葉綠素 a 與 b 加醋酸後，靜置 2 分鐘後以氮氣吹乾即可得到脫鎂脫植醇葉綠素 a 與 b。

(二) 人類血液淋巴球細胞的分離

淋巴球細胞是白血球的一種，主要執行人體內的免疫功能。健康的成年人白血球總數在 4~10×10^9/L 範圍內，其中嗜中性球最多，淋巴球次之，約佔 20-40%。淋巴球細胞主要來自骨髓幹細胞，當有抗原入侵時，淋巴細胞會去對抗抗原的入侵；再者，此細胞取得容易且細胞數多，因此本研究以淋巴球細胞作為氧化損傷保護的研究。

本實驗以真空採血管收集健康人體全血 2 毫升，並置於含有抗凝劑之採血管中，利用磷酸緩衝食鹽溶液(phosphate buffered saline；PBS)將全血做一比一的稀釋，以 Ficoll-Paque (Pharmacia Biotech)密度離心法，將轉速調整至 400G，離心 35 分鐘後分離出淋巴球並收集。經過兩次的 PBS 清洗後，加入適當的 RPMI1640 培養基調整細胞數。

二、分析項目與測量方法

(一) 葉綠素萃取物的純度分析

利用逆相高效液相色層分析(Reserved Phase High Performance Liquid Chromatography ；RP-HPLC)，以醋酸胺作為移動相(A solvent) (Almela, et al., 2000)，分析系統如下：

分析管柱：Spherisorb ODS-2 (250×40 mm), particle 5 µm (C_{18})

高壓幫浦：Waters Model 510

流速控制：Waters Model 680 自動梯度控制儀

注射器：Waters U6K

光譜偵測器：Fluorescence detection,

流動相 A：1M Ammonium acetate/methanol (2/8,v/v)

流動相 B：Acetone/methanol (2/8, v/v)

分析波長：激發波長 440nm，放射波長 660nm

葉綠素水萃取物的純度分析是利用其留滯時間(retention time)及光譜特性(visible absorption characteristics)確認。

(二) 細胞毒性實驗

本實驗是對淋巴球細胞添加不同濃度的葉綠素與 H_2O_2 作為氧化損傷的保護作用探討，但在探討葉綠素的保護功能之前，必須先進行細胞毒性試驗，以確保樣品在選取之反應條件（包括樣品濃度、反應時間和溫度）下，不會造成細胞死亡。樣品的細胞存活率愈高表示細胞毒性愈低，一般應於細胞存活率高於 90%的條件下繼續進行基因損傷之試驗。

本研究利用市售試劑組 CellTiter96®Aqueous One solution Cell proliferation 試劑組來測定人類淋巴球細胞在不同濃度的葉綠素水萃取物或過氧化氫下的存活率。利用[3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium;MTS]

與活細胞共同培養時，可被活細胞內具有活性的粒線體中 dehydrogenase 作用而切斷其 tetrazolium ring，形成 formazan，在 490nm 下可以測得最大吸光值(圖十四)。因此當吸光值較大時所代表活細胞數量愈多，故可作為偵測活細胞的指標(Cory, et al., 1991)。

圖十四：MTS tetrazolium 與其 formazan 產物的結構式

Figure 14: Structures of MTS tetrazolium and its formazan product.

將細胞均勻分到 24 孔洞的培養皿(24-well plate，約 2×10^5 cells/well)中，在 37℃下培養 24 小時後，再將各種的葉綠素水萃取物加入共同培養 30 分鐘；或是加入過氧化氫後培養 5 分鐘，同時利用 RPMI1640 培養基作為實驗的控制組。待達到時間點後將細胞以 PBS 清洗後，在適當的濃度下取出 100 µL 的細胞液放到 96 孔洞的培養皿(96-well plate)中，加入 20 µL 的 MTS 試劑，在 37℃下培養 4 小時，利用酵素免疫分析儀(Enzyme-Linked Immunosorbent Assay；ELISA)在 490 nm 測其吸光值，以檢測出細胞的存活率。當吸光值愈高表示活細胞數量越多，細胞存活率(survival)的計算方式為：處理組相對控制組的吸光強度百分比。

(三)清除 1,1-diphenyl-2-picryl-hydrazyl (DPPH)自由基能力的測定

一般抗氧化劑為氫原子供應者，在抗氧化的研究上可使用 DPPH 自由基來評估抗氧化物質提供氫原子的能力。因 DPPH 是較為安定的自由基，實驗上所採用的 DPPH 甲醇溶液為紫色，在 515 nm 下有強的吸光值，若與抗氧化劑結合，將會降低吸光值，由此藉以判斷樣本清除 DPPH 自由基的能力，其吸光值愈低，表示樣本清除 DPPH 自由基的能力愈強。本研究參考 Shimada 的方法，測定清除 DPPH 自由基的能力。 首先將葉綠素衍生物溶在乙醇溶液中，取出 0.4 mL 加入 0.8 mL 新鮮配置的 DPPH (10 mM)的甲醇溶液，均勻混合後靜置 30 分鐘，使用分光光度計(Spectrophotometer U-2000 Hitachi)檢測 515nm 的吸光值(Shimada, et al., 1992)。

清除率(scavenging effect)=[(控制組的吸光值 A_{515nm} − 樣品組的吸光值 A_{515nm})/ 控制組的吸光值 A_{515nm}]×100%

(四) H_2O_2 誘導 DNA 氧化損傷以及葉綠素的抗氧化研究

H_2O_2 在人體的代謝過程中會自然產生，若是 H_2O_2 在人體中累積到一定的量時，就會造成細胞 DNA 鹼基氧化作用而損傷，並造成 DNA 的斷裂(Wei, et al., 1999)，因此 H_2O_2 一般常用作氧化性 DNA 損傷的誘導劑(Halliwell and Gutteridge, 1999)。

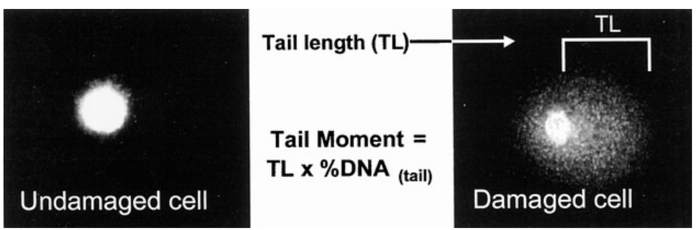
取適量培養於 RPMI 1640 培養基之細胞懸浮液，置於微量離心管中，在調整淋巴球細胞的數量後(2000 個 / mL) 分別加入不同濃度(5、20、50 μM)的各種葉綠素水萃取物，於 37℃下反應 30 分鐘，再加入 H_2O_2 使最後濃度成為 10μM 或 50μM，於 37℃下反應 5 分鐘，以離心的方式收集細胞，除去上層液，留下白色沉澱物，利用單細胞膠體電泳分析(彗星試驗分析)，以及測量 8-OHdG 來評估 DNA 損傷的程度。

(五) DNA 氧化損傷研究方法

目前有許多方法來量化 DNA 的氧化傷害，包括利用 HPLC 或氣相層析/質譜法(Gas Chromatography-Mass Spectrometry；GC-MS)測量鹼基的氧化產物。因為最容易受到傷害的鹼基是 Guanine (Halliwell, 1998)，當 DNAGuanine 的 C8 位置受到活性氧分子的攻擊而被氫氧化之後，即產生 8-OHdG，因此測量 8-OHdG 可作為 DNA 氧化傷害的指標。但是一般認為此方法用在 DNA 氧化損傷程度較低的情況，其正確性較高。此外，DNA 在萃取或水解的過程中，有時也會造成 DNA 進一步的氧化，例如，利用公牛胸腺萃取出的 DNA，利用酸水解並以 GC-MS 來測量 8-OHdG，比起利用酵素性水解後以 HPLC 測量所得到的 8-OHdG 值高(Lunec, 1998)；再者，DNA 在萃取的過程中，以 sodium iodide 來取代 phenol 會減少 8-OHdG 的

含量(Helbock, et al., 1998)。

為解決上述 DNA 在分離或水解過程中因氧化所造成測量 8-OHdG 含量的誤差，Ostling 和 Johnson (1984) 提出利用微小膠體電泳法(microelectrophoresis)偵測單一細胞 DNA 的斷裂情形，其優點是可以在螢光顯微鏡下直接觀察單一細胞 DNA 損傷的情況；Singh 等人(1988)建立鹼性微小洋菜膠電泳法(alkaline microgel electrophoresis)，即所謂的單細胞膠體電泳法(single cell gel electrophoresis；SCGE)，又簡稱為彗星分析(comet assay)，其中將 DNA 雙股解開的步驟在鹼性的環境中進行，因此可以偵測到細胞 DNA 的單股斷裂(single-strand breaks；SSBs)(圖十五)。其原理為細胞 DNA 受到傷害而斷裂時，斷裂的片段因為分子較小，因而在電泳槽中藉由正負極電流的牽引而移動，染色後顯現的暈點如同彗星般拖出長長的尾巴。相對於未受傷的 DNA 因為分子較大而在電泳槽中不會移動，因此藉由移動的距離來評估 DNA 損傷的程度，目前以利用尾動量(tail moment) 參數來評估 DNA 損傷最為普遍(Olive, et al., 1990)。



圖十五：在螢光顯微鏡下所觀察到 DNA 未損傷(左圖)，以及損傷(右圖)的情形，並以尾動量(Tail Moment)來表示受損傷程度

Figure 15 : Under a fluorescence microscope, the comet-like images resulting from the extension of DNA were scored as a reflection of the single-strand breaks.Tail moment was defined as follows: TM = TL × % DNA

(六) 單細胞膠體電泳法(彗星分析)

1. 電泳膠片製備

取正常溶點的洋菜膠溶液(normal melting gel agarose)，注入玻片上，立即蓋上蓋玻片置於 4℃ 5 分鐘，待凝固後移除蓋玻片，此為第一層。

取出先前於 37℃下反應的各實驗組細胞溶液，加入低溶點的洋菜膠溶液(low melting gel agarose, LMA)，趁凝固以前快速注入玻片上，立即蓋上蓋玻片置於 4℃ 5 分鐘，待凝固後移除蓋玻片，此為第二層，此時細胞已經存在於此層中。

將製備好的玻片浸入細胞水解液（2.5 M NaCl, 100 mM Na-EDTA, 10mM Tris, pH=10, 1% sodium sarcosinate, 1% Triton X-100 及 10% DMSO），於 4℃下反應 1 小時使細胞膜破裂。

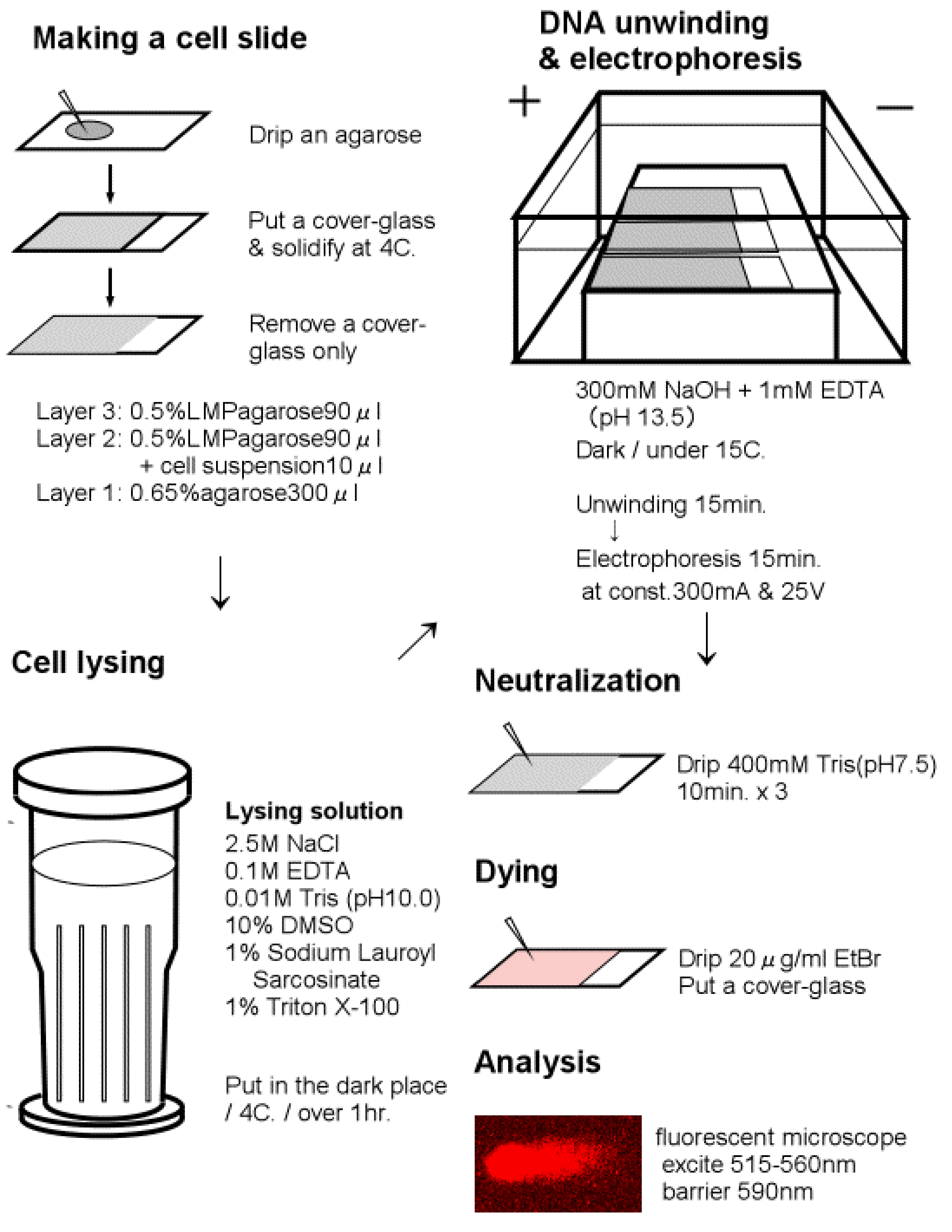
2. DNA 解雙股以及電泳

將玻片由水解液中取出，以二次水清洗後，置於水準式電泳槽中，加入電泳液使玻片於鹼性的電泳液中解開雙股螺旋的結構(unwinding)。待 20 分鐘後，開啟電泳電源，調整電流為 300 mA，電壓保持在 25V 左右，時間為 30 分鐘。電泳結束後以 Tris buffer 中和。

3. DNA 氧化損傷分析

玻片以 ethidium bromide 染色後，使用螢光顯微鏡，在 400 放大

倍率下觀察，以彗星分析系統 VisCOMET®分析，每個葉綠素純化物質濃度做二重複，每個玻片分析 20 個細胞 Comet 圖案亮度，以尾動量(Tail Moment)來表示 DNA 受損傷程度。有關利用彗星分析 DNA 損傷的流程圖如圖十六。



圖十六：彗星分析的流程圖

Figure 16：Protocol of comet assay

(六) 8-氫氧 2'-去氧鳥糞核糖核苷(8-hydroxy-2- deoxyguanosine；8-OHdG)測定

1. DNA 的抽取分離

為保持 DNA 的完整性，在低溫且避光線的環境下進行 DNA 的抽取分離，以避免 DNA 斷裂或降解。DNA 的分離是利用 Genomic DNA-purification kit (Bertec Enterprise, Taiwan)。各實驗組的淋巴球細胞經過不同葉綠素濃度的處理，以及被 H_2O_2 所誘導氧化損傷後，分裝在 1.5 mL 的微量小管中，離心收集下層的細胞。接著以 100 µL 的 PBS 將細胞洗出，隨後加入 700 µL extraction solution 將細胞溶出。加入 4 µL proteinase K，置於 56℃水浴機中直到細胞完全破裂(約 1-3 小時)，之後移至室溫。加入 700 µL 的 DNA binding buffer，搖晃均勻 5 分鐘使雜物凝集。

將所有的溶液倒入spin column，再將 spin column 套入collection tube，以 12-14000 rpm 離心 1 分鐘。將洗下來在 collection tube 中的液體倒掉，在 spin column 中加入 700µL 的 wash solution 清洗（此步驟重複二至三次），此時 DNA 仍存在於 spin column 的膜層中。將 spin column 套在新的無菌微量小管，置入 60℃的烘箱約 5-10 分鐘，讓 Ethanol 蒸發。加入 50 µL，60-70℃的 elution solution 到 spin column，停留約 2-3 分鐘，讓 DNA 溶出，再以 6000 rpm 離心 2 分鐘，並重複

2 次收集所有的 DNA，保存 DNA 於-20℃備用。

2. DNA 的定量

DNA 在 260nm 處有最大的吸光值，蛋白質則在 280nm 處有最大的吸光值。因此，可以用 260nm 波長進行分光測定 DNA 濃度，1 OD 約為 50 μg/mL 的雙股 DNA。取出 50 μL DNA 溶液稀釋於理想的濃度後放入比色管中，使用分光光度計(Spectrophotometer U-2000 Hitachi)檢測 260 nm 的吸光值即可計算出樣品稀釋前的濃度：

DNA（μg/mL）=50×OD260 讀數×稀釋倍數

為確認 DNA 的純度是否受到蛋白質的殘留，而影響實驗，同時以分光光度計(Spectrophotometer U-2000 Hitachi)檢測 280 nm 的吸光值，純淨 DNA 的 OD260 nm /OD280 nm 為 1.8，若 260 nm/280 nm = 1.8 (1.6-2.0) 表示純化過程正常；若 260 nm/280 nm＜1.8 表示蛋白質太多沒有去除乾淨；若 260 nm/280 nm＞1.8 表示有 RNA 的污染。

3. 8-OHdG 的測定

8-OHdG 的測定是利用競爭型 ELISA 的方法進行分析(Japan Institute for the Control of Aging, Japan)。首先將 8-OHdG monoclonal antibody 以 PBS 稀釋，接著將已經 coating 8-OHdG 的 96 孔 ELISA 盤取出，接著將已經混合好 50 μL 的樣品或 8-OHdG 標準溶液(0.5, 2,

8, 20, 80 ,200ng/mL)與 50 μL 的 8-OHdG 抗體注入每個孔中，蓋上蓋子且用膠帶封緊每個邊後，於 37℃下反應 1 小時。此時細胞 DNA 萃取物中的 8-OHdG 就會與 coating 8-OHdG 一起競爭 8-OHdG 抗體。若細胞 DNA 萃取物中的 8-OHdG 愈多，則 coating 8-OHdG 與 8-OHdG 抗體結合在 96 孔 ELISA 盤的量就愈少。

1 小時後將內容物倒掉，加入 250 μL 的 washing 溶液到本次實驗的每個孔洞中，輕輕左右搖晃培養皿後倒掉。倒置培養皿並用乾淨的面紙吸乾殘存的 washing 溶液，重複二次。此時細胞 DNA 萃取物中的 8-OHdG 與 8-OHdG 抗體結合物洗掉，留下 coating 8-OHdG 與 8-OHdG 抗體結合在 96 孔 ELISA 盤中。

再將 100 μL 的二次抗體到本次實驗的每個孔洞中，蓋上蓋子且用膠帶封緊每個邊後，於 37℃下反應 1 小時，目的是將二次抗體與 8-OHdG 抗體結合。1 小時後將內容物倒掉，加入 250 μL 的 washing 溶液到本次實驗的每個孔洞中，輕輕左右搖晃 ELISA 盤後倒掉。倒置 ELISA 盤並用乾淨的衛生紙吸乾殘存的 washing 溶液，重複二次。

加入 100μL 的 chromatic substrate 3,3’,5,5’-tetramethylbenzidine (呈色劑)到本次實驗的每個孔洞中，以鋁箔紙包起避光，在室溫下反應 15 分鐘。最後加入 100μL 的 phosphoric acid 終止呈色反應，利用 ELISA reader 於 450 nm 測量吸光值。以標準 8-OHdG 的濃度製作出

標準曲線(含線性迴歸方程式,Y=mx+b)後，算出各實驗組中 8-OHdG 的含量。8-OHdG 的含量以 ng /μg DNA 表示。

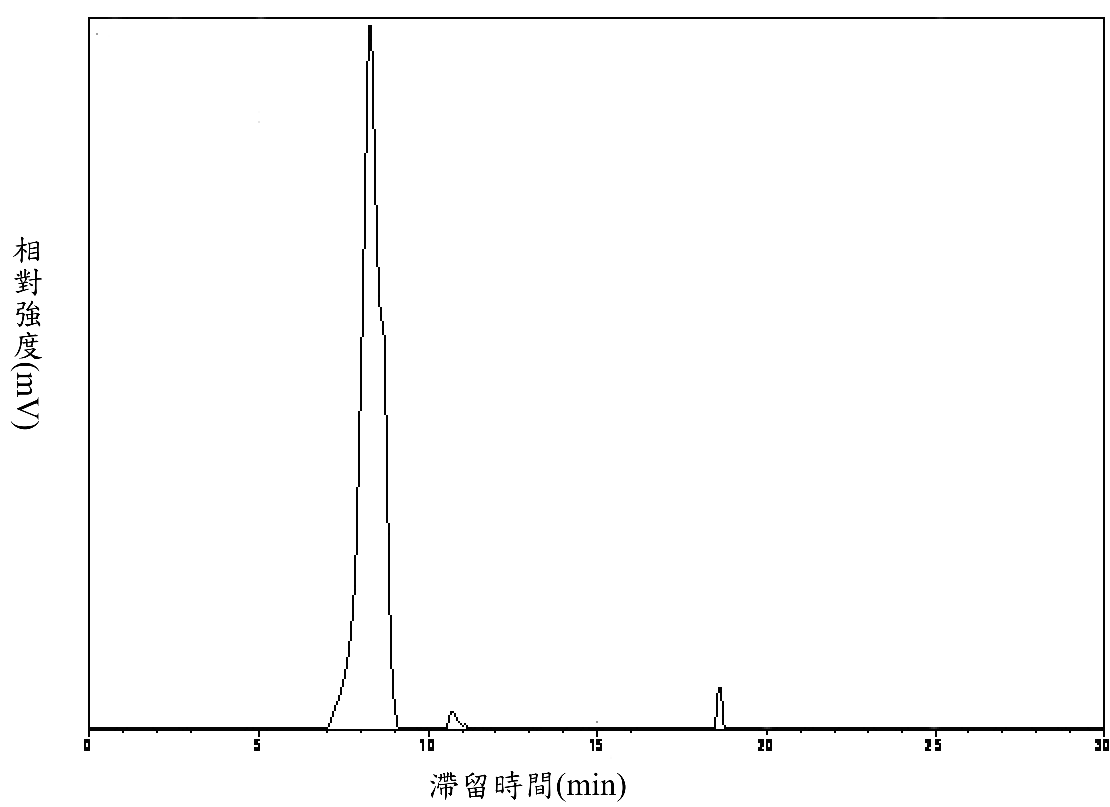
三、統計分析方法

本實驗數據以 SPSS® 10.0 版統計軟體進行分析，以 Mean±SD 表示，並以 ANOVA 做變異分析，並以 Duncan's Multiple Range test 作各組差異比較，$P<0.05$ 作為統計上的差異。

第二節　結果

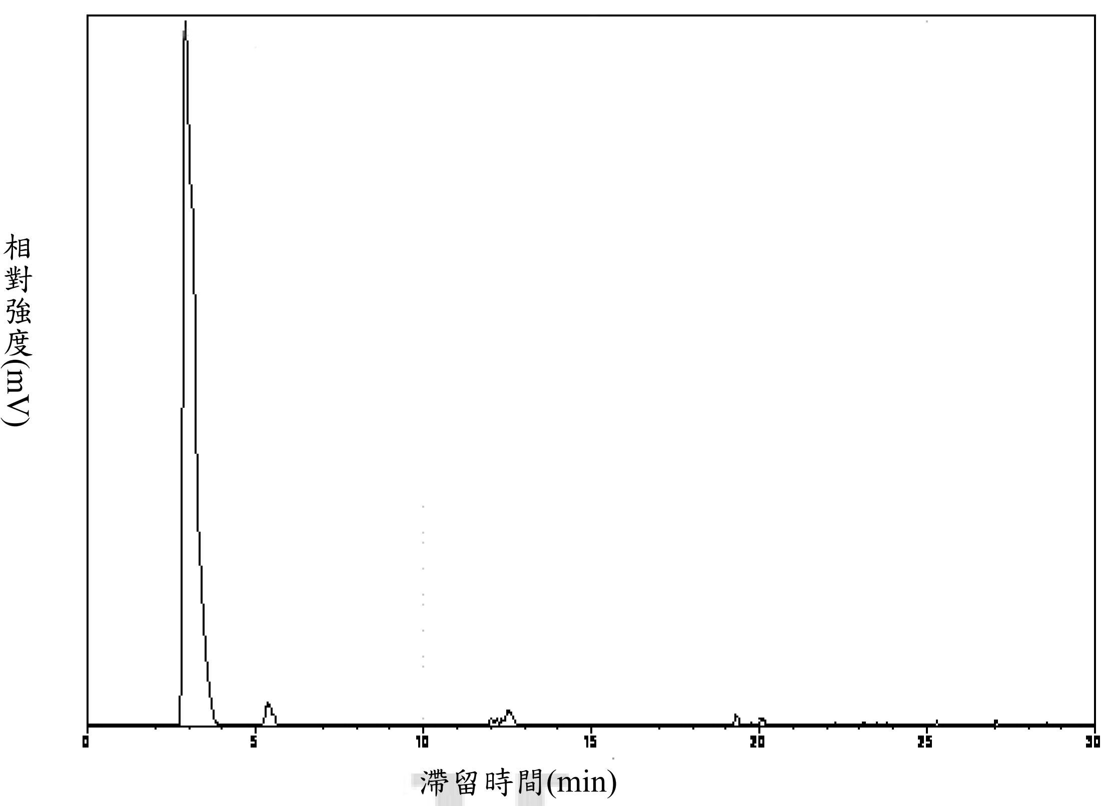
一、葉綠素水萃取物純度分析

所有葉綠素水萃取物經過 RP-HPLC 的分析，其純度均達 99％以上(圖十七~圖二十)。



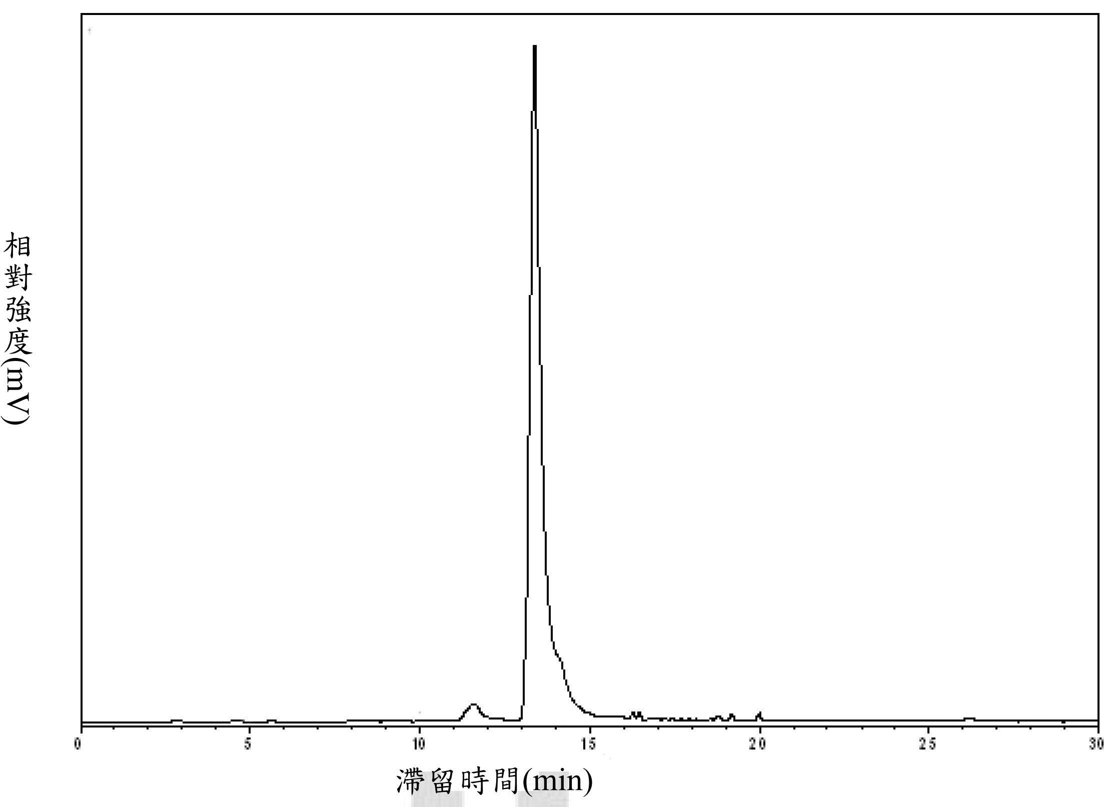
圖十七： 葉綠素水萃取物-Chlorophyllide a 成分之 RP-HPLC 分析圖

Figure 17：Elution profile of the chlorophyllide a by RP-HPLC.



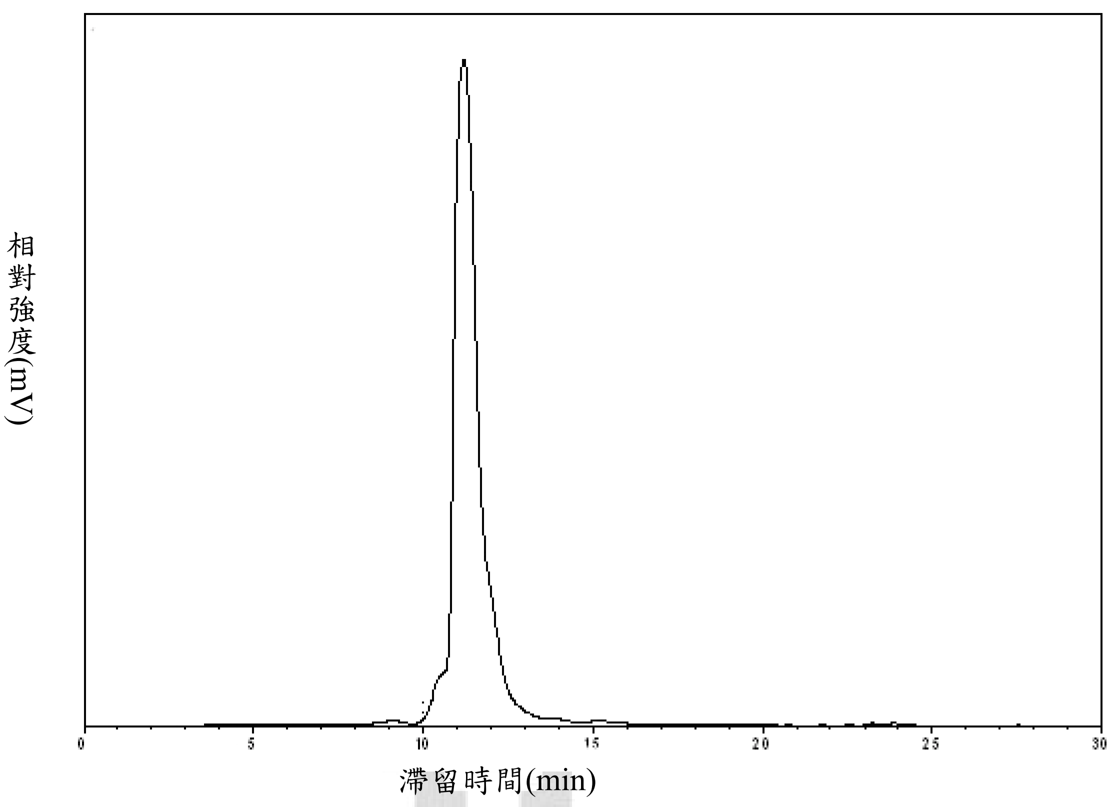
圖十八 葉綠素水萃取物-Chlorophyllide b 成分之 RP-HPLC 分析圖

Figure 18：Elution profile of the chlorophyllide b by RP-HPLC.



圖十九 葉綠素水萃取物-Pheophorbide a 成分之 RP-HPLC 分析圖

Figure 19：Elution profile of the Pheophorbide a by RP-HPLC.



圖二十 葉綠素水萃取物-Pheophorbide b 成分之 RP-HPLC 分析圖

Figure 20：Elution profile of the Pheophorbide b by RP-HPLC.

二、細胞存活率分析

由表三的結果顯示，人類淋巴球細胞在添加不同種類與濃度的葉綠素水萃取物(5、20、50μM) 30 分鐘與 H_2O_2(0、10、50μM) 5 分鐘後細胞的存活率。其中以添加 50μM Pho b 的細胞活率最高(99.8±2.6) ，而添加 Pho b 與 50μM H_2O_2 的細胞活率最低(95.2±2.8)。然而所有組別當中並沒有統計上的差異，且細胞存活率都達到 95％以上。表四顯示細胞在無添加葉綠素的情形下給予 10μM 或 50μM 的 H_2O_2 來誘導 DNA 的氧化傷害，經過 5 分鐘的共同培養之後，亦沒有明顯造成細胞死亡的現象。因此，後續實驗則利用上述濃度的各種葉綠素水萃取物作為本研究的對象。

表三：人類淋巴球細胞加入葉綠素水萃取物與 H_2O_2 培養的存活率 [1]

Table 3 Effect of chlorophyll derivatives and H_2O_2 on human lymphocyte viability[1]

	lymphocyte viability (%)		
Chlorophyll derivatives	5μM	20μM	50μM
Chlorophyllide a	97.5±6.4	98.4±5.3	96.2±4.3
Chlorophyllide a + 10μM H_2O_2	96.8±2.5	97.8±3.8	97.5±2.4
Chlorophyllide a + 50μM H_2O_2	95.8±3.2	96.1±2.5	97.1±3.4
Chlorophyllide b	98.8±2.2	96.8±3.5	99.2±4.4
Chlorophyllide b + 10μM H_2O_2	97.±4.2	97.9±3.8	98.1±4.5
Chlorophyllide b + 50μM H_2O_2	96.±2.8	96.9±3.3	96.5±2.5
Pheophorbide a	95.8±6.5	96.9±3.4	99.4±2.5
Pheophorbide a + 10μM H_2O_2	96.8±3.5	97.7±3.2	95.7±4.2
Pheophorbide a + 50μM H_2O_2	95.2±2.4	96.5±2.8	96.3±3.2
Pheophorbide b	98.4±1.6	99.7±3.3	99.8±2.6
Pheophorbide b + 10μM H_2O_2	97.2±2.6	96.5±4.3	97.2±1.6
Pheophorbide b + 50μM H_2O_2	95.2±2.8	95.1±0.3	98.1±2.5
Chlorophyllin	98.5±2.3	96.4±2.2	95.4±6.1
Chlorophyllin + 10μM H_2O_2	96.0±0.3	97.8±1.2	96.8±2.1
Chlorophyllin + 50μM H_2O_2	96.7±2.3	99.1±0.9	97.5±3.8

[1] Mean±SD, Viability (measured by the MTS assay) was determined both prior to (100%) and following chlorophyll derivatives and H_2O_2.

表四：人類淋巴球細胞加入 H_2O_2 培養後的存活率 [1]

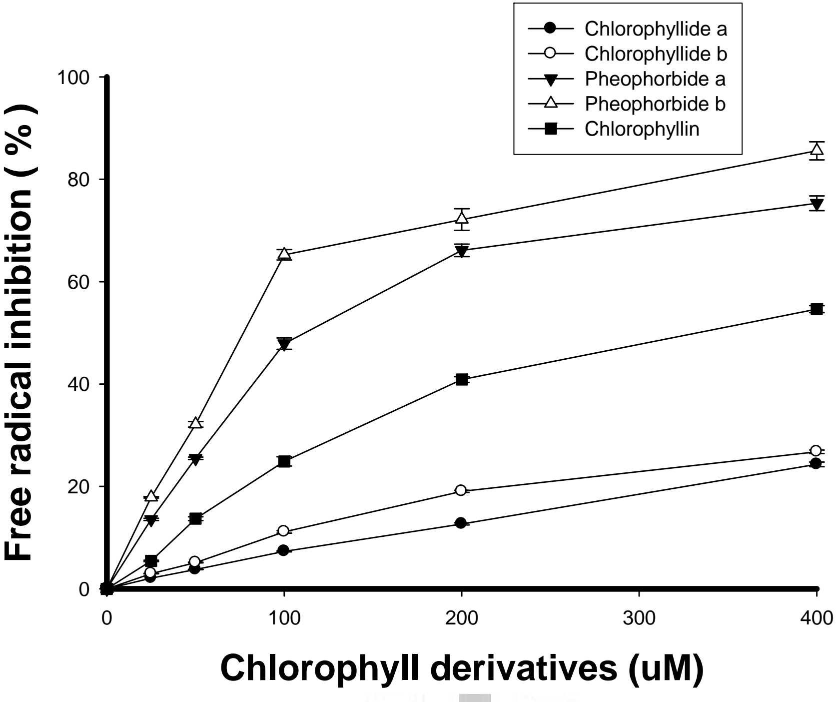
Table 4 Effect of pretreated H_2O_2 on human lymphocyte viability[1]

	lymphocyte viability (%)	
	10μM	50μM
H_2O_2	99.4±2.4	98.2±7.1

[1] Mean±SD, Viability (measured by the MTS assay) was determined both prior to (100%) and following H_2O_2 treatment.

三、清除 1,1-diphenyl-2-picryl-hydrazyl (DPPH)自由基能力

圖二十一顯示葉綠素水萃取物對於清除 DPPH 自由基的能力。清除自由基的能力由強至　依次為 Pho b＞Pho a＞Chlin＞Chlide b＞Chlide a。如果以葉綠素的種類區分時: Pho＞Chlin＞Chlide，且 b form＞a form。其中達到 50%清除 DPPH 自由基(IC50)所需要的葉綠素濃度分別是 Pho b：75 µM，Pho a：120 µM,，Chlin：360 µM，Chlide a，Chlide b：＞800 µM (表五)。



圖二十一：葉綠素水萃取物清除 DPPH 自由基的能力。

Figure 21：DPPH-scavenging capacity of chlorophyll derivatives. The absorbance inhibition for DPPH was monitored at 515 nm. Results represent the mean±SD　(　:Chlorophyllide a,　: Chlorophyllide b,　: Pheophorbide a,　: Pheophorbide b,　: Chlorophyllin.)

表五：清除 50%的 DPPH 自由基(IC50)所需要的葉綠素濃度

Table 5: DPPH radical scavenging effect of chlorophyll derivatives calculated in terms of IC_{50}

Treatment	IC_{50}(μM)
Chlorophyllide a	＞800
Chloropyhllide b	＞800
Pheophorbide a	120
Pheophorbide b	75
Chlorophyllin	360

四、H_2O_2 誘導 DNA 氧化損傷

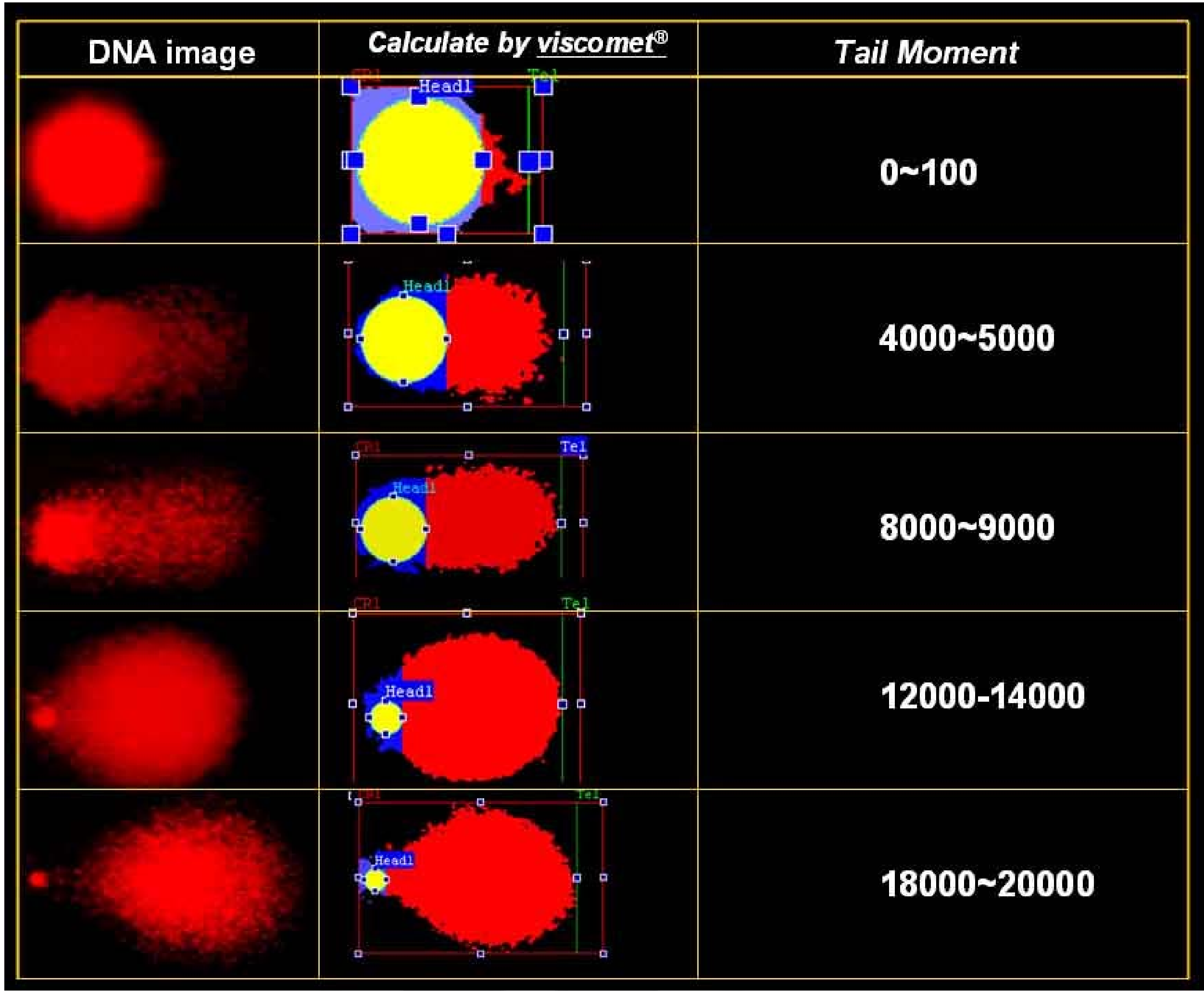
本實驗的 計將人類淋巴球細胞在 95%的細胞存活率下，檢 DNA 的氧化損傷情形。由表四可看出細胞在不同濃度的 H_2O_2 作用下，都保持穩定的存活率，但在這樣的濃度下是否會誘導 DNA 氧化損傷，利用單細胞膠體電泳法直接在電子顯微鏡下測量 DNA 的斷裂程度，或是測量 DNA 氧化產物 8-OHdG 的含量，即可驗證 H_2O_2 在人類淋巴球細胞中是否造成 DNA 損傷。表六顯示淋巴球細胞 DNA 損傷的程度，以彗星分析之平均尾動量而言，10μM H_2O_2 之氧化損傷較控制組增加 60 倍(尾動量 100 vs 6386）；50μM H_2O_2 之氧化損傷較控制組增加 150 倍(尾動量 100 vs 14537) 。圖二十二顯示在螢光顯微鏡下所呈現之 DNA 斷裂彗星分析電泳圖，以以及利用 VisCOMET® 軟體分析所得的結果。此外，測量 DNA 氧化損傷產物-8-OHdG 的含量，發現 10μM H_2O_2 之 8-OHdG 較控制組增加 4 倍(0.55 vs 2.30）；50μM H_2O_2 之氧化損傷較控制組增加 7 倍(0.55 vs 3.98)。因此無論利用彗星分析 DNA 斷裂或測量氧化代謝產物，均顯示淋巴球細胞之 DNA 損傷，都隨著 H_2O_2 暴露濃度的增加而提高。

表六：H_2O_2 對於人類淋巴球細胞氧化損傷的影響

Table 6. Effect of H_2O_2 upon human lymphocyte DNA damage[1]

	DNA damage	
	Comet assay[2]	8-OHdG(ng /μg DNA)
H_2O_2 10μM	6386±803[a]	2.30±0.13[a]
H_2O_2 50μM	14537±1692[b]	3.98±0.33[b]
Control	100±21[c]	0.55±0.06[c]

[1]Mean±SD; Values featuring different letters differ significantly as regards H_2O_2 levels (ANOVA $p < 0.05$),
[2]Mean tail moment (TM) was calculated for the comet assay.



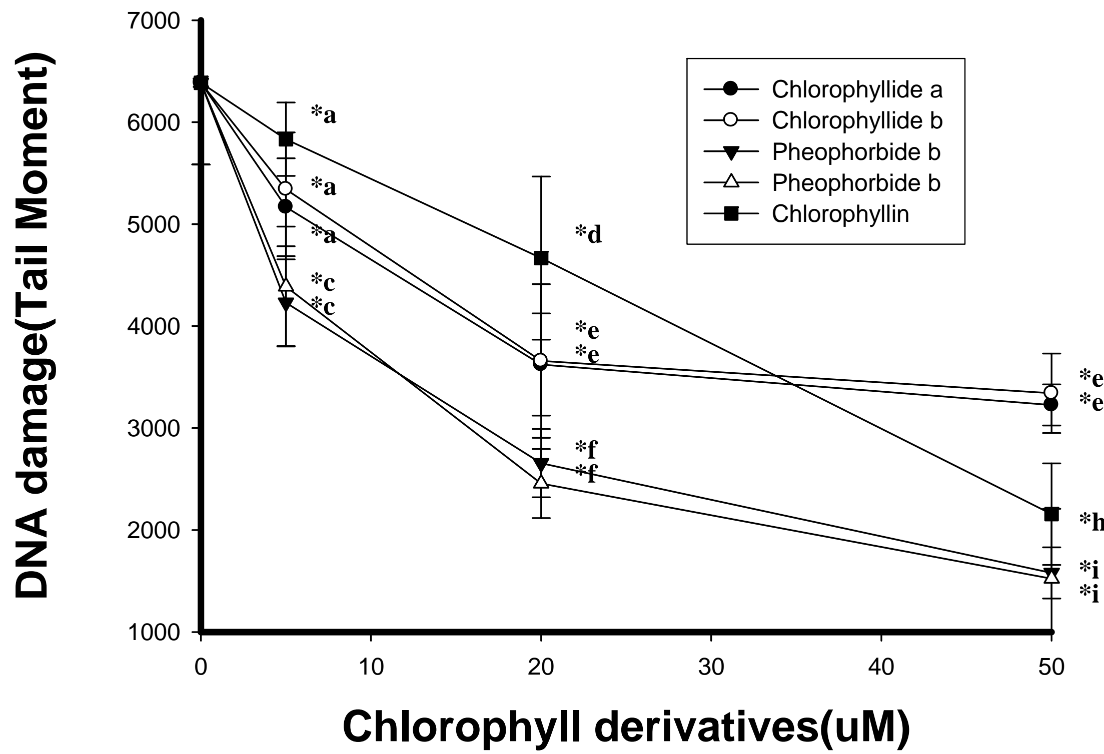
圖二十二：以彗星分析 DNA 損傷的結果

Figure 22：The tail moment (integrated value of DNA density multiplied by the DNA migration distance) was used as the primary measure of DNA damage

五、葉綠素對於 H_2O_2 誘導淋巴球細胞 DNA 氧化損傷的影響

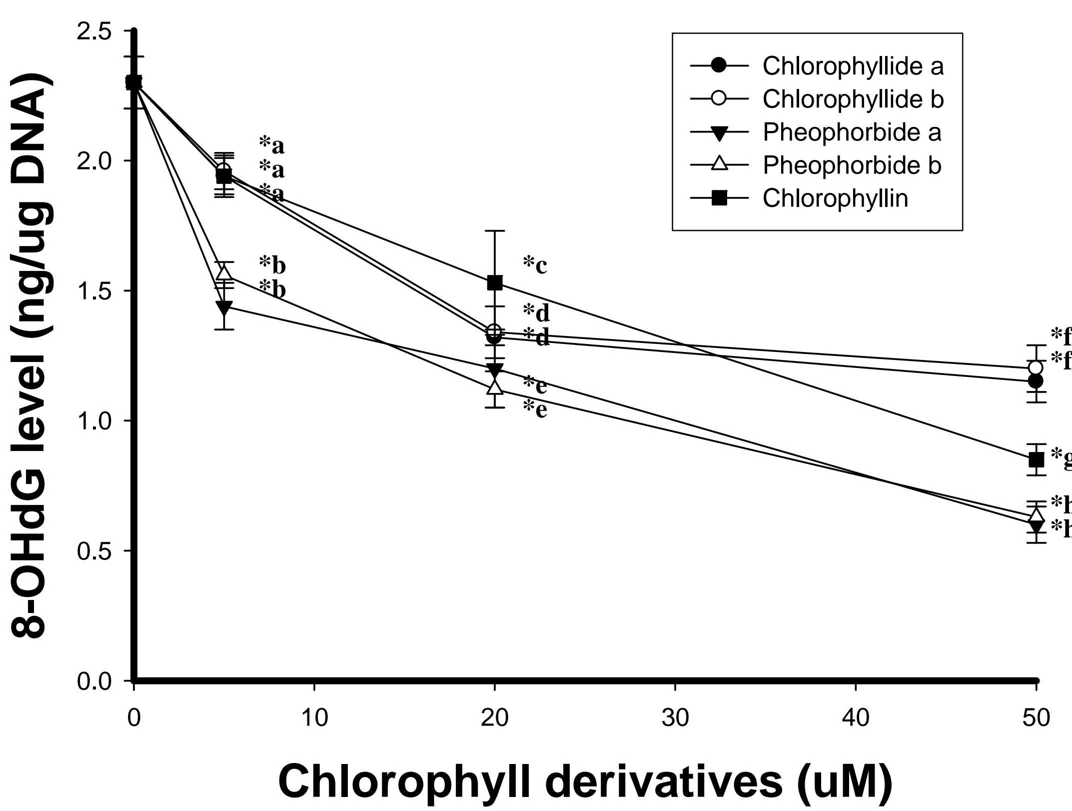
圖二十三與圖二十四顯示添加各種不同濃度的葉綠素水萃取物質後，再給與 10μM H_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果。葉綠素水萃取產物在 a form 與 b form 之間都沒有統計上的差異，但是無論是添加何種類型的葉綠素水萃取物質或是銅鈉葉綠素，其細胞氧化損傷都隨著濃度的增加而減少，顯示這些物質藉由抗氧化作用減少 DNA 的氧化傷害，其中又以脫去鎂離子的 Pho 效果最好(與對照組作比較時，Pho a 與 b 的抑制效果分別為 75.28% 和 76.17%)。

圖二十五及二十六顯示細胞在接受高濃度 50μM 的 H_2O_2 後各種葉綠素的保護效果。細胞的氧化傷害增加，然而 Chlide 或 Chlin 都無法對細胞進行保護作用，僅 Pho 降低細胞的氧化傷害(與對照組作比較時，Pho a 與 b 的抑制效果分別為 19.63% 與 20.18%)。



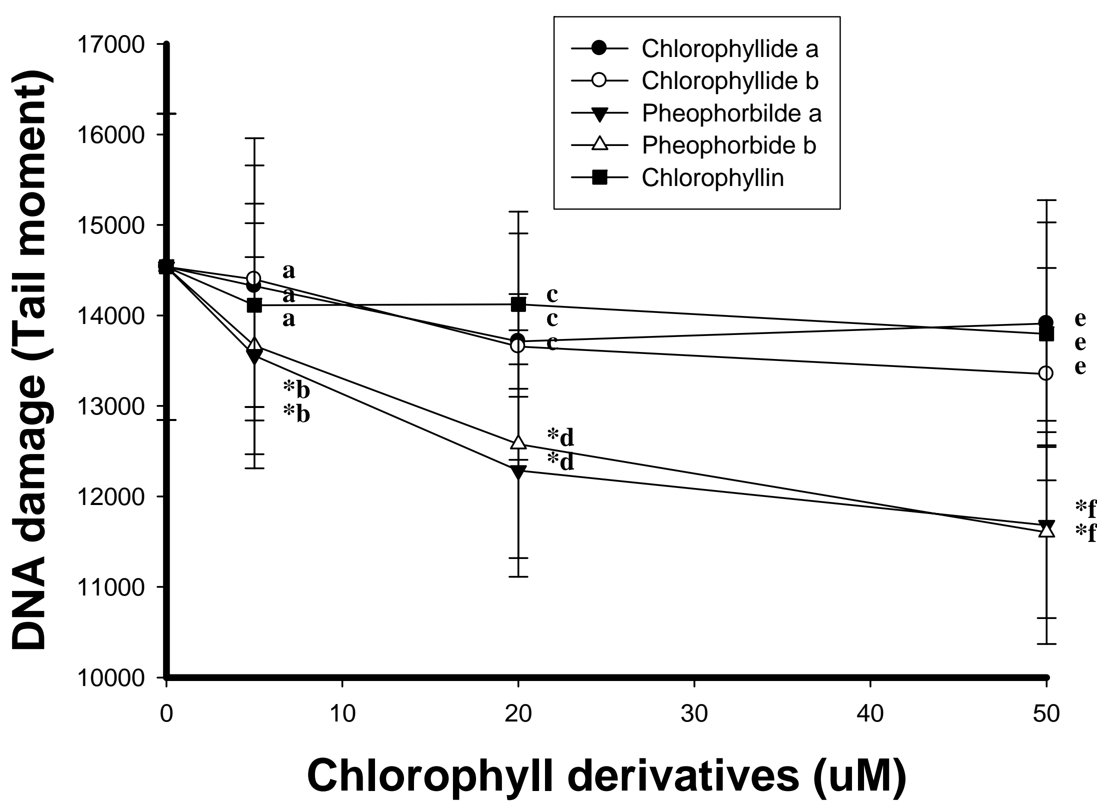
圖二十三：添加各種不同濃度的葉綠素水萃取物質後再給與 10μM H_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(彗星分析)

Figure 23: Effect of Chlororphyll derivatives pretreatment upon 10μM hydrogen peroxide-induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the comet assay in lymphocyte DNA. Results are the mean±SD. (●:Chlorophyllide a, ○: Chlorophyllide b, ▲: Pheophorbide a, △: Pheophorbide b, ■: Chlorophyllin.) Values with different letters differ significantly as regards oxidative damage when comparing between different chlorophyll derivatives (ANOVA, $p < 0.05$); * $p < 0.05$ refers to differences in oxidative damage as compared with the control (without chlorophyll derivatives).



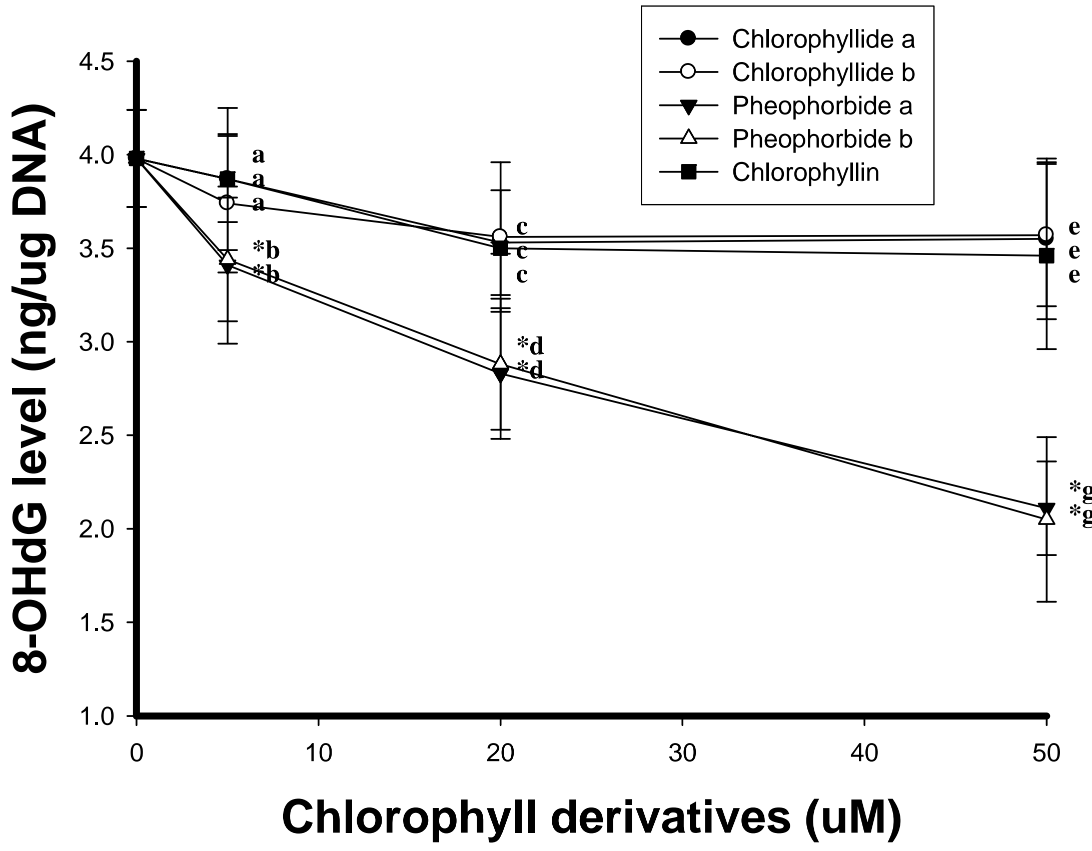
圖二十四：添加各種不同濃度的葉綠素水萃取物質後再給與 10µM H_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(8-OHdG)

Figure 24: Effect of chlorophyll derivatives pretreatment upon 10µM hydrogen peroxide-induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the 8-OHdG levels in lymphocyte DNA. Results are the mean±SD. (●:Chlorophyllide a, ○: Chlorophyllide b, ▲: Pheophorbide a, △: Pheophorbide b, ■: Chlorophyllin.) Values with different letters differ significantly as regards oxidative damage when comparing between different chlorophyll derivatives (ANOVA, $p < 0.05$); * $p < 0.05$ refers to differences in oxidative damage as compared with the control (without Chlorophyll derivatives).



圖二十五：添加各種不同濃度葉綠素水萃取物質後再給與 50μMH_2O_2 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(彗星分析)

Figure 25: Effect of chlorophyll derivatives pretreatment upon 50μM hydrogen peroxide-induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the comet assay in lymphocyte DNA. Results are the mean±SD. (●:Chlorophyllide a, ○: Chlorophyllide b, ▲: Pheophorbide a, △: Pheophorbide b, ■: Chlorophyllin.) Values with different letters differ significantly as regards oxidative damage when comparing among different chlorophyll derivatives (ANOVA, $p < 0.05$); *p<0.05refers to differences in oxidative damage as compared with the control (without chlorophyll derivatives).



圖二十六：添加各種不同濃度的葉綠素水萃取物質後再給與 $10\mu H_2O_2$ 所誘導氧化損傷的人類淋巴球細胞 DNA 損傷的結果(8-OHdG)

Figure 26: Effect of chlorophyll derivatives pretreatment upon 50μM hydrogen peroxide-induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the 8-OHdG levels in lymphocyte DNA. Results are the mean±SD. (●:Chlorophyllide a, ○: Chlorophyllide b, ▲: Pheophorbide a, △: Pheophorbide b, ■: Chlorophyllin.) Values with different letters differ significantly as regards oxidative damage when comparing among different chlorophyll derivatives (ANOVA, $p < 0.05$); * $p < 0.05$ refers to differences in oxidative damage as compared with the control (without chlorophyll derivatives).

第三節 討論

一、植物性化學物質抗氧化性的比較

1954 年自由基的發現，證實與許多慢性疾病有關；長期暴露在含氧的自由基環境中將導致氧化壓力。一般生物體內的抗氧化系統與活性氧是維持在一個平衡的狀態，當體內活性氧分子多時，它們將破壞細胞中的蛋白質、脂質甚至 DNA，最後造成細胞的老化或死亡(Sies, 1986； Halliwell and Gutteridge, 1999)。

許多天然的植物性食物(phytochemicals)或維生素都是抗氧化物的最佳來源。由於近年來，一些蔬果中的植物性化學物質，例如：葉綠素、類胡蘿蔔素、維生素 C、維生素 A 以及維生素 E 等物質，在正常的攝取下具有抗氧化、抗突變性等保健功能。Ong 等學者在 1989 年的研究發現在上述的這些植物性化學物質中，葉綠素能發揮最佳的抗突變性表現。此外，Kamat 等學者 2000)的研究指出葉綠素的抗氧化效果比維生素 C、glutathione 或 mannitol 來的好。

二、葉綠素在體內的代謝

葉綠素主要以兩種形式存在於新鮮的蔬菜水果中，分別是 chlorophyll a 與 b (Almela, et al., 2000)，以新鮮菠菜為例， hlorophyll a 與 b 的含量比約為 2.5：1 (Ferruzzi, et al., 2001) 。但是隨著綠色蔬菜種植的老化，葉菜中的 hlorophyllase 的濃度隨之增加，使葉綠

素脫去非極性的植醇鏈而產生極性較高的脫植醇葉綠素(Chlide) (Trebitsh, et al., 1993）。此外，當它們在酸性環境下，例如葉菜類食物在烹調、製備或加工等過程中加入酸性物質例如醋；或是攝取食糜後進入胃與胃酸混合之後，會失去其葉綠素中心 orphyrin 環內的鎂離子而轉變成脫鎂葉綠素(Phe），或脫鎂脫植醇葉綠素 Pho)。Ferruzzi 等學者(2001) 利用模擬人體小腸 Caco-2 細胞模式的研究發現，Phe 可以經由人類體內的乳糜微粒吸收進入循環系統。動物的研究發現 (葉, 2003），這些葉綠素的代謝產物透過門靜脈到達肝臟後， 乎轉變成 Chlide 或 Pho。因此本實驗以 Chlide 或 Pho 等葉綠素的代謝產物來進行研究，除可以比較各種葉綠素衍生物之抗氧化能力外，還可以藉此模擬葉綠素代謝物質對人體所發揮的抗氧化效力。

三、葉綠素含量對人體淋巴球細胞的影響

Frenzilli 等學者(2000) 曾針對銅鈉葉綠素(Chlin)以及過氧化氫等 18 種化合物，添加在人類淋巴球細胞後，探討劑量對其細胞存活率的影響；研究顯示要達到 95％以上的細胞存活率時，添加於細胞中的葉綠素或過氧化氫的最大濃度分別為 200 μM 與 50 μM。此研究結果與本實驗表三與表四所呈現細胞的存活率一致。此外，Frenzilli 等學者(2000)也分析淋巴球細胞 DNA 的損傷程度，發現 DNA 的氧化損傷隨著細胞添加過氧化氫濃度增加而增加，此結果與本實驗中表五

DNA 氧化損傷的結果一致。

本實驗對人體淋巴球細胞(2000 個/毫升)所使用的葉綠素最高濃度為 50 μM。若是依據健康的人體中淋巴球細胞約有 1.5×10^{7} 個來換算，約需要 0.3 公克的葉綠素　能在血液中達到相對濃度。惟目前缺　葉綠素生體可利用率的相關資料。根據 Ferruzzi 等學者(2001) 的研究指出：約有 5-10%經過乳糜化的葉綠素可以進入人類 Coca-2 小腸細胞，估算人體約需從飲食中攝取 3~6 公克的葉綠素。一般新鮮菠菜中平均大約含有 2% 的葉綠素(López-Ayerra, et al., 1998)，因此推估在理想的狀態下至少需攝取約 300 公克的新鮮菠菜。此項結果　合行　院衛生署建議每日蔬菜攝取量。惟上述的推估是在最理想的轉換率下計算而得，有關食物從攝取進而達到人體內細胞的實　含量，必須經過更進一步研究，包括葉綠素的生體可利用率、或是葉綠素代謝產物的轉換率等，　可確認。

四、銅鈉葉綠素的抗氧化作用

銅鈉葉綠素廣泛的用於各種的成藥、食品中，因此與其相關的抗氧化、抗腫瘤研究亦是備受注目。Kumar 等學者在 1999 年第一位提出 Chlin 抗氧化的量化研究；發現當　plasmid DNA 暴露在 6 Gy 的 γ 輻射線照射下會產生氧化傷害而造成單股斷裂，若 DNA 在照射前先添加不同濃度的 Chlin (10μM~100μM) ，其 DNA 的氧化傷害保護效

果隨著 Chlin 劑量的增加而有顯著的效果 (22.6~68.1%的抑制 DNA 單股斷裂) ，接著利用 pulse radiolysis 來實　測量 Chlin 捕捉自由基的能力亦發現：這些自由基 (hydroxyl radical、deoxyribose peroxyl radical、singlet oxygen) 與 Chlin 結合的比例隨著劑量的增加而提高，且具有相當高的反應常數 (rate constant) (Kumar, et al., 1999；Kamat, et al., 2000) 。

此外，利用 Electron Spin Resonance (ESR)來分析 Chlin 與 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical 以及 5,5-dimethyl-1-pyrrolidine-N-oxide with hydroxyl radical 等自由基的化學反應能力時，　實 Chlin 具有捕捉各種自由基的能力(Kumar, et al., 2001），此結果與本實驗中葉綠素清除 DPPH 自由基的結果是一致的，惟過去學者主要是針對 Chlin 的抗氧化性，本研究更進一步的探討其他存在自然界中葉綠素的水萃取衍生物清除自由基的能力，發現 Pho a 及 b 的效果比 Chlin 強。

在活體外(ex vivo)的研究上發現：Chlin 可以抑制老鼠肝臟粒線體中放射現所誘導的脂質過氧化情形；研究顯示 50μM 的 Chlin 的添加抑制了 53% 的 thiobarbituric acid reactiove substance (TBARS) 與 30.6% 的 lipid hydroperoxides 等自由基的產生；同時其抗氧化酵素 SOD 與 GSH 的含量也相對的比未添加 Chlin 時來的高；若是以相同

濃度的 Chlin 與維生素 C、mannitol 以及 tert-butanil 來比較對老鼠肝臟粒線體的抗氧化能力時，Chlin 的抗氧化能力最強，且　乎是維生素 C 的 2 倍。由此可知 Chlin 可以保護粒線體的細胞膜來對抗放射線所造成的氧化傷害(Kamat, et al., 2000)。

五、吡啉類(porphyrins)的抗氧化作用

葉綠素的基本結構為四個　yrrole 所組成的吡啉環(porphyrins)，其中共有　　個共軛雙鍵(conjugated double bounds)。若與類胡蘿蔔素相比較，植物黃體素(lutein)與玉　黃素(zeaxanthin)含有 9 個共軛雙鍵；茄紅素與胡蘿蔔素含有 11 個共軛雙鍵，隨著共軛雙鍵數目增加其抗氧化性隨之增加，吡啉環中共有 11 個共軛雙鍵，因此具有極佳的　inglet oxygen 或自由基反應結合能力。

從脂質自動氧化(autoxidation)的研究中亦發現：將葉綠素添加在油脂中，其過氧化價(peroxide value, POV)明顯的比未添加葉綠素低；且由 ESR 的分析證實有葉綠素 π 陽離子自由基(π-cation radical)的存在，顯示葉綠素在油脂氧化反應中容易失去電子而形成 tetrapyrrole radical catio　，同時這些電子可以提供給自由基來終止連鎖的過氧化作用(Endo, et al., 1985)。

除了葉綠素之外，其他吡啉環結構的化合物也被證實可以形成 π 陽離子自由基，例如 tetraarylporphyrin (Borg, et al., 1970)、

bacteriochlorophyll 、bacteriopheophytin (McElroy, et al., 1974）、鐵離子或是銅離子的吡咻環 (Ravikanth, et al., 1994）、 離子的吡咻環 (Renner and Fajer, 2001)以及其他類吡咻環結構的化合物等(Jayaraj, et al., 1996 ；Scheidt, et al., 1996），因此可以合理的去證實葉綠素的代謝產物中，只要具有吡咻環結構都可以藉由以下的反應機制來達成安定自由基目的(Endo et al. 1985)：

ROO· + Chlorophyll → ROO：( ) Chlorophyll·(+)

ROO：( ) Chlorophyll·(+) + ROO· → Inactive products

(ROO·; peroxyl radical)

六、天然葉綠素代謝產物的抗氧化比較

從上述的討論中發現，帶有吡咻環結構是葉綠素最主要抗氧化的位置，不過其 部分的化學結構的差異亦會對葉綠素的抗氧化性產生影響。Lanfer-Marquez 等學者(2005)利用 -胡蘿蔔素 色消失的速度 (β-carotene blenching method)，來測定葉綠素代謝產物的抗氧化能力。結果發現：帶有 基 (-CHO) 的葉綠素代謝產物 Phe b 比帶有甲基 ($-CH_3$) 的 Pho a 更有較強的抗氧化能力)。這樣的結果與本實驗利用 DPPH 自由基的清除效果相比是一致的。

在本研究中，更進一步的探討各種葉綠素代謝產物在細胞內的抗氧化能力表現，發現無論是用顯微鏡測量 DNA 氧化傷害斷裂的情

況，亦或是測量氧化傷害的代謝產物 8-OHdG 時，Chlide a 與 b 之間，以及 Pho a 與 b 之間是沒有顯著的差異的，是否 a 、b 結構在體內的細胞穿透力上不同，或是在細胞中代謝的途徑不同，則需進一步的研究來證實。

在葉綠素的吡咻環結構中，是否有螯合離子或是不同種類的離子螯合在其中間，亦會對葉綠素的抗氧化性產生影響，從本研究中發現：所有葉綠素的衍生產物對低濃度的 H_2O_2 都具有保護的效果，但對於高濃度下的 H_2O_2 則僅 Pho 仍能抵抗細胞的氧化傷害；且從 DPPH 的實驗中也證實 Pho 的抗氧化作用最強，其次是 Chlin，最後是 Chlide。若從這些葉綠素代謝產物的化學結構來分析：Chlide 含有鎂離子；Chlin 含有銅離子而 Pho 的結構中是不含任何金屬離子。過去的研究發現：吡咻環結構中具有螯合離子的能力(Longo, et al., 1973)；過去在中研院植物所的研究中，曾對其他四種極性較低的葉綠素衍生物在螯合亞鐵離子的能力上做比較時發現：與 Pho 同樣未帶有鎂離子的脫植醇葉綠素(Phe)，在螯合亞鐵離子的能力表現上比帶有鎂離子的葉綠素高(unpublished data)。推測在本研究中，淋巴球細胞在添加 Pho 之後，除了吡咻環結構可安定細胞中 H_2O_2 自由基之外，還具有較佳的能力去螯合亞鐵離子，進而減少亞鐵離子在細胞中產生 Fenton 作用(圖二)，使金屬離子誘導的氧化傷害降到最低。

第四章　葉綠素的水萃取衍生物對黃麴毒素所誘導肝細胞 DNA 損傷之影響

第一節　研究方法

本研究的主旨為探討葉綠素及其衍生物對於黃麴毒素所誘導的肝臟細胞 DNA 損傷的影響。由於過去研究顯示，含銅鈉葉綠素可以有效的抑制 DNA 突變，因此本研究以 Chlin 為對照實驗，與其他的葉綠素水萃取物作比較。

一、實驗材料

綠素水萃取物的製備：同前述

二、分析項目與測量方法

(一) 細胞株的選取

本研究以 Hepa-1c1c7 (Hepa-1)細胞作為研究對象，細胞株來自於食品工業研究所生物資源保存及研究中心(Bioresource Collection and Research Centre Taiwan).。Hepa-1 細胞為小鼠肝腫瘤細胞(mouse hepatoma)，可培養在 90% Dulbecco’s Modified Eagle Medium (DMEM)與添加 10%　牛血清的培養基中，並培養在 37 °C 溫度與 5% CO_2/95% 的空氣中。相關的研究指出，此細胞株能穩定的表現細胞色素 P450-1A 的活性，且對於 AFB_1 有高度敏感的細胞毒性(Karenlampi, 1987)。

(二) 黃麴毒素的來源與濃度的選

黃麴毒素 B_1(AFB_1)購自 sigma 公司，將購得之黴菌毒素，配置的方法是將適量的 dimethyl sulfoxide (DMSO)溶劑注入 AFB_1 的　品中，搖晃使黴菌毒素的結　溶解成 50 mg/mL 的濃度，之後再以蒸水稀釋，並以微量離心管分裝，置於-20℃　箱保存備用。

由於 Hepa-1 細胞株對 AFB_1 有高度敏感的細胞毒性，在培養 48 小時後，細胞的　數致死量(LD50)為 68 ng/mL (Karenlampi, 1987)，為了使本實驗的細胞維持在 95％以上的存活率，因此 AFB_1 所選取的濃度為(0、5 ng/mL、10 ng/mL)，培養時間為 48 小時。

(三) 細胞毒性實驗

本實驗是以 Hepa-1 細胞添加不同濃度的葉綠素作為對抗 AFB_1 損傷的保護的探討，但在探討葉綠素的保護功能之前，必須先進行細胞毒性試驗，以確保樣品在選取之反應條件（包括樣品濃度、反應時間和溫度）下，不會造成細胞死亡。樣品的細胞存活率愈高表示細胞毒性愈低，一般應於細胞存活率高於 90%的條件下繼續進行基因損傷之試驗。

本研究利用市售試劑組 CellTiter96®Aqueous One solution Cell proliferation 試劑組來測定 Hepa-1 細胞在不同濃度的葉綠素水萃取物或過氧化氫下的存活率。測定方法與步驟同第二章第一節。

(四) 黃麴毒素誘導 DNA 損傷以及葉綠素的抗基因毒性研究

過去的研究指出葉綠素對於細胞的抗突變作用主要是經由分子捕捉(molecular trapping)的能力。由於葉綠素具有 porphyrin 的化學結構，可藉由分子平面結構的　水作用力(hydrophobic interactions)的作用與致突變物質形成複合體，因而降低了致突變物質與細胞中 DNA 結合而造成的突變傷害(Hayatsu, 1992 ; Dashwood and Liew, 1992)。但是這樣的分子捕捉能力顯示在細胞內或細胞外並無法得知，即使葉綠素能進入細胞內，是否能調控肝細胞的解毒酵素，進而減少 DNA 的傷害亦無法得之，因此　計了以下的實驗。

本實驗　計將 Hepa-1 細胞進行 96 小時的細胞培養實驗，分別於 0 、24、 48 、72 小時更換培養液，以 PBS 清洗細胞二次。首先 Hepa-1 細胞於 0 、24 小時添加不同濃度(0、5、20、50μM)的葉綠素水萃取物(Chlide a, Chlide b, Pho a, Pho b, or Chlin)共同培養之後，分為二大組: 第一組將 Hepa-1 細胞在第 48 小時、72 小時添加黃麴毒素(0 ng/mL, 5 ng/mL, or 10 ng/mL)與葉綠素水萃取物共同培養; 第二組將 Hepa-1 細胞在第 48 小時後以 PBS 清洗細胞並不再添加葉綠素水萃取物(washout variation)，只在第 48 小時、72 小時單　添加黃麴毒素(0 ng/mL, 5 ng/mL, or 10 ng/mL)，其目的是想藉此　解先前 48 小時葉綠素水萃取物的保護效果是否能已進入細胞內做調控。

(五). Aflatoxin B_1-DNA adducts 的分析

1.細胞中 DNA 含量的測定

DNA 含量的測定是以 PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, MN)試劑組來分離與測定。將肝細胞以 trypsin-EDTA 打下收集,離心後的細胞以 PBS 清洗,加入 3 mL 的 cell Lysis 溶液注入已經抽乾的細胞培養皿中，用定量 管上下吸使細胞均勻的裂解，之後將所有細胞裂解液移至 15mL 的塑膠試管中。

加入 15 μL 的 RNAse A 溶液並均勻搖晃 25 次，在 37℃下 15-60 分鐘後將樣本置於室溫中冷卻。加入 1 mL protein precipitation 溶液高速 20 ，離心 2000x g 10 分鐘。

倒出含有 DNA 的上清液進入含有 3mL 100% isopropanol 搖晃 50 次，接著離心 2000x g 3 分鐘，DNA 沉澱出現。倒掉上清液後以面紙吸 多 的水後加入 3 mL 的 70% ethanol 倒轉試管數次清洗 DNA。

加入 15mM Na_2CO_3: 30mM $NaHCO_3$ (pH9.6) 37℃ 2 小時使沉澱粒全溶解，加入 2-3 倍體積的 95% 精，使 DNA 沉澱、離心並乾燥,並利用第二章第一節中 述 DNA 定量的方法測出 DNA 的含量，最後將 DNA 的濃度以 hydration solution 調整成 0.5μg/μL (25μg/ 50 μL)，三重 ，貯存於-20℃備用。

2. Hepa-1 細胞中 AFB_1-DNA adducts 的測定

AFB_1-DNA adducts 的測定是利用競爭型 ELISA 的方式進行分析，　明如下：

(1). Imidazole ring-opened AFB_1-DNA adducts 的合成

本實驗檢測肝細胞中的 AFB_1-DNA adducts ，所以必須先合成已知量的 imidazole ring-opened AFB_1-DNA adducts (iro-AFB_1-DNA)，作為待測樣品的競爭性抗原。其合成來自於長庚大學醫學系謝玲玲博士的研究室，其步驟如下：將已加熱變性的牛胸腺 DNA 加入磷酸鈉的溶液中，並加入已溶解於 di-chloromethane 的黃麴毒素後，再加入 m-chloroperoxy benzoic acid，於適溫下　　25 小時。接著加入等量的 chloroform，以去除　未反應遊離態的 AFB_1，接著加入 95%乙醇，使 DNA 沉澱後離心。為了將 AFB_1-DNA adduct 上的 imidazole 環打開，再加入 $Na_2CO_3/NaHCO_3$ 溶液。將合成的 iro-AFB_1-DNA，以吸收光譜 260nm 測其 DNA 的含量，再以吸收光譜 360nm 測量 AFB_1 的含量，最後調整為每百個核甘甘酸上鍵結 2 個 AFB_1。

(2). 將抗原　上 96 孔 ELISA 盤 (coating ELISA plate)

在 96 孔 ELISA 盤上，　除外圍一　外，其　每一孔中加入 100μL 的 iro-AFB1-DNA adducts solution(5ng/100μL)。進入 37℃恆溫箱烘乾

至少 24 小時備用。

取出 96 孔 ELISA 盤並以 PBS-Tween(0.1%)將每一孔內未吸

部分清洗乾淨。加入 200 μ

l 1% FCS PBS Tween 溶液於每一孔中，進入 37℃恆溫箱烘乾

1 小時，以便 蓋孔 上未吸 的 iro-AFB_1-DNA adducts 空 ，降

低非特異性抗原-抗體的作用。最後再以 PBS-Tween 將每一孔內清

洗乾淨。

(3). 抗體(6A10)的配製

利用謝玲玲博士所研發成功的「抗 iro-AFB1-DNA adduct」單株

抗體 6A10 來測量 AFB_1-DNA adducts 的含量(Hsieh and Hsieh,

1993)。

(4). 競爭性抗原(Hepa-1 DNA adducts)或標準濃度的 iro-AFB_1-DNA

的配製

為製作標準曲線，因此取 160 µL 的標準濃度(10、25、50、100、

250、500、1000 fmole) 與 160 µL 的 6A10 抗體混合均勻，以每個孔

注入 100 µL 的體積，分別加入 3 個孔中(三重)，置於 37℃恆溫箱

反應 90 分鐘。另外取 320 µL 的 Hepa-1 細胞的 DNA 樣本及 320 µL

的 6A10 抗體混合均勻，以每個孔注入 100 µL 的體積，分別加入 6

個孔中(六重)，置於 37℃恆溫箱反應 90 分鐘。接著加熱 90℃、15

分鐘將 DNA 變性後，立刻　浴 10 分鐘，以 PBS-Tween 將 well 內清洗乾淨。

(5).加入第二次抗體

加入 conjugated 二次抗體，以 1：1500 的比例稀釋於 1% FCS PBS Tween 中，每個孔加入 100 μL，置於 37℃恆溫箱反應 90 分鐘。以 PBS-Tween 將每一孔內清洗乾淨。最後利用 0.01M 的 diethanolamine　洗 ELISA 盤二次。再加入 100 μL 的受酶質(alkaline phosphatase substrate)，並放入恆溫箱使其呈色，最後以波長 405nm 的 ELISA 判讀器(ELISA reader E1-340 Bio-Tek Instruments)測定孔中的受質呈色。

(6). 計算公式

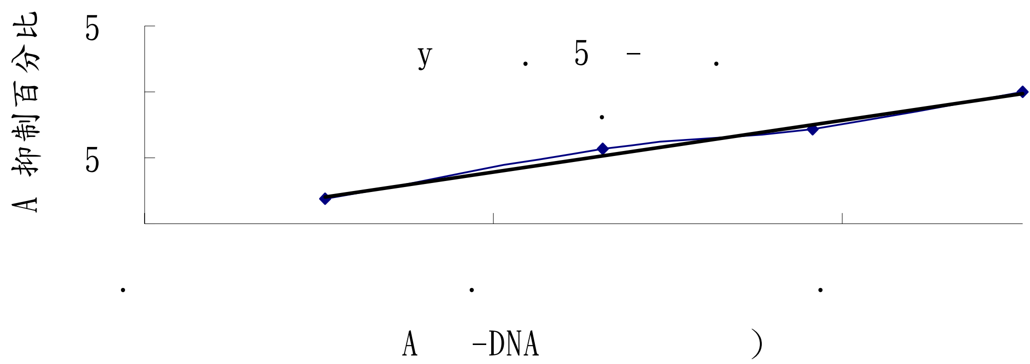
標準濃度:

100%-(standard 反應劑量呈色/零反應劑量呈色×100%)

受測樣本:

100%-(sample 反應劑量呈色/未 treatment 反應劑量呈色×100%)

先利用線性回歸的方式算出 AFB_1-DNA adducts 標準曲線(圖二十七)，並以線性回歸的公式求出 Hepa-1 細胞中 AFB_1-DNA adducts 的含量。



圖二十七：AFB_1-DNA adducts 的標準曲線圖

Figure 27: Standard curve of AFB_1-DNA adducts

(六). Glutathione-S-transferase(GST)活性分析

1.細胞中蛋白質的含量

蛋白質濃度依 Bradford (1976)之方法定量。先以不同濃度 BSA[100 mg/mL(for stock-20℃) 1、 0.5、 0.25、 0.125、 0.0625、 0 mg/mL] 建立標準曲線，再取 20μL 細胞均質液加入 140 μL 的水，放在 96 well 的細胞培養皿中。Bio Rad 蛋白質分析溶液 (Bio-Rad protein assay dye R-250)以 PBS 稀釋 5 倍,每 well 再加入 200 μL 混合均勻，以分光光度計測量波長 595 nm 的吸光值，再依標準曲線計算蛋白質濃度(mg/mL)。

2. Glutathione-S-transferase 活性的測量

測量 GST 活性是利用 Calbiochem® GST assay kit 試劑組來測定，其原理是利用 GST 酵素會催化 GSH 的硫醇基(-SH) 轉至 1-chloro-2,4-dinitrobenzene (CDNB)的反應(圖二十八)，所產生的物質在波長 340 nm 下具有吸收值(ε = 9.6 mM-1cm-1) ，每單位時間內增加的吸收值代表其反應物的生成速率，繼而計算 GST 的比活性(specific activity)。

首先取出 40 μL 的細胞液加入 160 μL 含有 BSA 的 potassium phosphate buffer 稀釋後，取出 20 μL 放入分光光度計的比色管中，接著加入 360μL 含有 CDNB/potassium phosphate buffer 的混合液與

$$GSH + Cl\text{-}C_6H_3(NO_2)\text{-}NO_2 \xrightarrow{GST} GS\text{-}C_6H_3(NO_2)\text{-}NO_2 + Cl^- + H^+$$

圖二十八： GSH 與 CDNB 的反應

Figure 28: The reaction of CDNB with GSH

20μL 的 GSH　分混合均勻後，以分光光度計在波長 340 nm 的吸光值下進行　　(time scan)5 分鐘，並測量波長與時間　的　率。當率愈大時表示其 GST 的活性愈強。

計算方式如下:

1.Net Rate: (ΔA340nm/min)= $Slope_{Hepa\text{-}1\ 細胞樣本}$- $Slope_{Blank}$

2.GST 的活性(mU/mL)= (Net Rate/0.0096)×稀釋的倍數

(U=mol/min, 0.0096= GSH-CNDB extinction coefficient)

3.最後以每個樣本中蛋白質含量做校正，將 Hepa-1 細胞中 GST 的活性(mU/mL)除以細胞中總蛋白質的含量(mg/mL) ，單位以 mU/mg 蛋白質來表示。

三、統計分析方法

本實驗數據以 SPSS® 10.0 版統計軟體進行分析，以 Mean±SD 表示，並以 ANOVA 做變異分析，並以 Duncan's Multiple Range test 作顯著性差異比較，$P<0.05$ 作為統計上的差異。

第二節 結果

一、細胞存活率分析

由表七的結果顯示，Hepa-1 細胞在添加本實驗所用最高濃度的葉綠素水萃取物(50μM)後細胞的存活率與控制組比較都沒有顯著的差異。當細胞在 48 小時候給予不同濃度的 AFB_1(0、5 ng/mL、10 ng/mL)後的存活率也都與控制組之間沒有統計上的差異。且研究顯示所有組別當中的細胞存活率都達到 95%以上。因此，本實驗可利用上述濃度的各種葉綠素水萃取物以及 AFB_1 作為本研究的對象。

表七 Hepa-1 細胞株對葉綠素水萃取物與 AFB_1 培養後的存活率 [1]

Table 7. Effect of Chlorophyll derivatives and AFB_1 on Hepa-1 cell viability.[1]

	Hepa-1 cell viability (%)		
	AFB_1 (ng/mL)		
Chlorophyll derivatives	0	5	10
Control	100	98.66±0.56	97.05±0.89
Chlorophyllide a	101.50±0.68	101.84±0.89	103.23±0.47
Chlorophyllide b	103.78±1.02	98.92±1.22	106.06±0.89
Pheophorbide a	102.11±1.45	98.86±0.87	105.74±3.45
Pheophorbide b	96.86±2.11	97.13±1.47	105.11±1.02
Chlorophyllin	97.77±1.57	95.66±1.23	95.79±2.11

[1] Mean±SD relative to control (no chlorophyll derivative treatment).Cells were treated for 48 h with 50 μM of each chlorophyll derivative, then treated with the chlorophyll derivative and AFB_1 for another 48 h, after which an MTS assay was performed at 96 h to determine viability.

二、黃麴毒素 B_1 誘導 DNA 損傷

本實驗的　計在 Hepa-1 細胞株在 95%的細胞存活率下，DNA 的損傷研究。表八顯示 Hepa-1 細胞株在受到 AFB_1 培養後，AFB_1-DNA adducts 的含量，其中添加 5 ng/mL AFB_1 之後其 AFB_1-DNA adducts 的含量比未添加時增加 12 倍(0.15 vs 1.8)；添加 10 ng/mL 黃麴毒素 B_1 之後其氧化損傷比未添加時增加 17.5 倍(0.15 vs 2.63)。這樣的結果顯示隨著細胞中 DNA 的損傷隨著添加黃麴毒素 B_1 濃度的增加而提高。

表八：AFB_1 對於 Hepa-1 細胞株 DNA 損傷的影響 [1]

Table 8. Effect of AFB_1 upon Hepa-1 DNA damage[1].

	AFB_1-DNA adducts
AFB_1 5 ng/mL	1.80±0.16
AFB_1 10 ng/mL	2.63±0.16
Control	0.15±0.07

[1] Mean±SD. Cells were treated for 48 h with 5,10 ng/mL AFB_1 to determine AFB_1-DNA adducts.

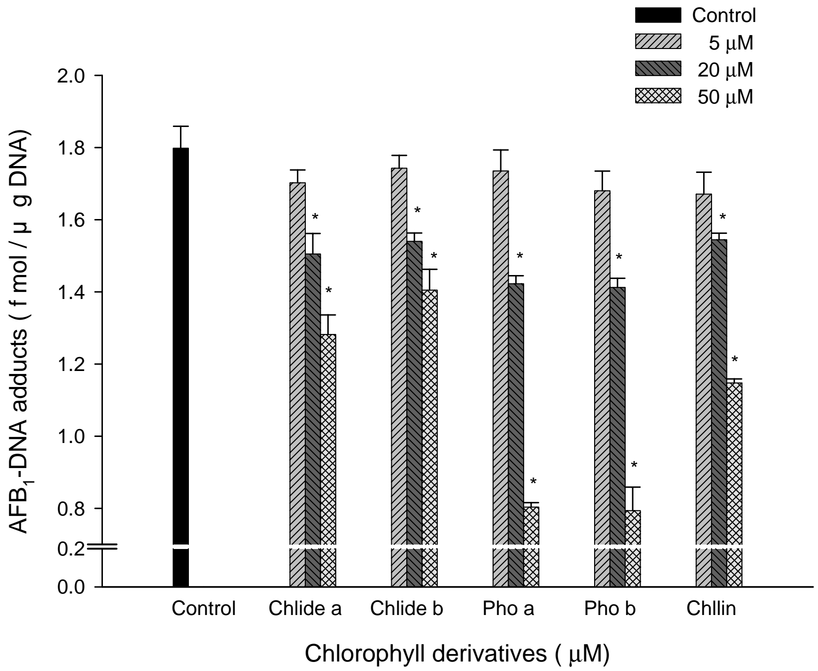
三、葉綠素水萃取物對於 Hepa-1 細胞株 AFB_1-DNA adducts 產生的影響

圖二十九為 Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後再給與 AFB_1 所誘導 DNA 損傷的結果，其中以未添加葉綠素僅添加 5 ng/mL 的 AFB_1 做為控制組。結果顯示低濃度的葉綠素水萃取物(5μM)與控制組間並沒有顯著的差異，表示低濃度的葉綠素水萃取物(5μM)對 AFB_1 誘導 Hepa-1 細胞株的傷害並無保護的作用。但是隨著添加葉綠素水萃取物的濃度增加(20μM、50μM)，其保護的作用就明顯的上升，其中以葉綠素的種類區分，AFB_1-DNA adducts 含量由少而多依次 Pho＜Chlin＜Chlide，而天然葉綠素水萃取物中 a form 與 b form 之間並無統計上的差異；尤其是添加 50μM 的 Pho a 或 Pho b 之後的細胞，其 AFB_1-DNA adducts 的含量僅是未添加時的 0.45 倍 (Pho a: 0.82 vs 1.8, Pho b: 0.81 vs 1.8)。顯示其保護的效果最佳。

圖三十顯示細胞在接受高濃度的 AFB_1 誘導 DNA 損傷的結果。其中以未添加葉綠素僅添加 10 ng/mL 的 AFB_1 做為控制組。結果顯示低濃度的葉綠素水萃取物(5μM)與控制組間仍然沒有顯著的差異，而添加 10μM 的葉綠素水萃取物與控制組間 AFB_1-DNA adducts 的含量有顯著的差異，惟葉綠素水萃取物各組之間並沒有統計上的差異性；隨著葉綠素水萃取物濃度的增加，在添加 50μM 的葉綠素水萃取

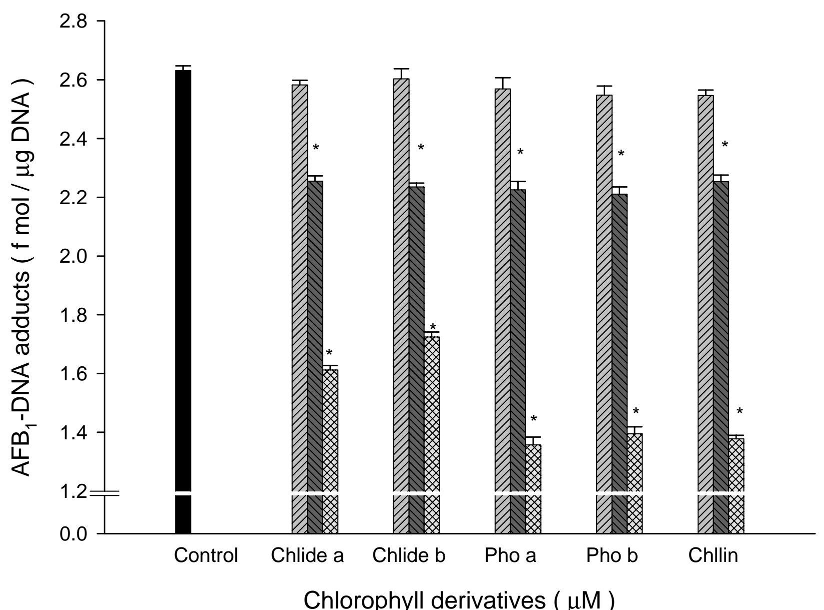
物後，AFB_1-DNA adducts 的含量明顯的下降更低，其中以 Pho a 與 b，以及 Chlin 的效果大於 Chlide。

圖三十一與圖三十二為 Hepa-1 細胞株在添加 5 ng/mL(圖三十一)以及 10ng/mL(圖三十二)黃麴毒素 B_1 前清除所有葉綠素水萃取物(washout) 之後所測得 AFB_1-DNA adducts 含量的結果。研究發現無論是高濃度或低濃度的黃麴毒素 B_1，添加 Chlide 48 小時後去除，其所測得的 AFB_1-DNA adducts 含量與對照組間並無統計上的差異，顯然 Chlide 的添加無法預防黃麴毒素 B_1 對 Hepa-1 細胞株的傷害；而在 Pho 與 Chlin 組別中所測得 AFB_1-DNA adducts 的含量與對照組之間有統計上的差異，顯然 Pho 與 Chlin 雖沒有直接接觸黃麴毒素 B，但仍然對 Hepa-1 細胞發揮保護的作用。



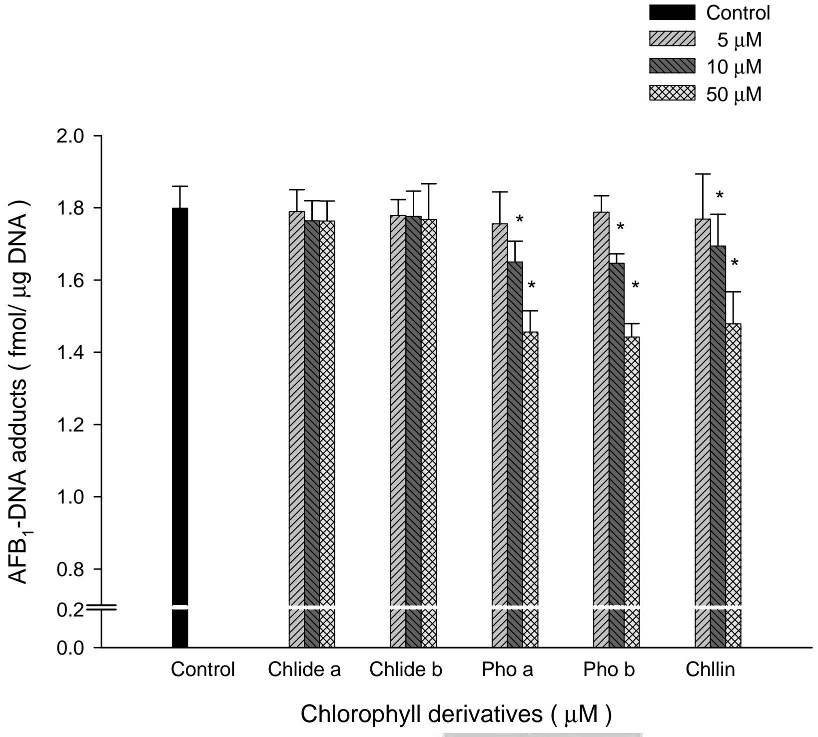
圖二十九：Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後再給與 5 ng/mL AFB_1 所誘導 DNA 損傷的結果

Figure: 29 Effect of pretreatment with chlorophyll derivatives before challenge with 5 ng/mL AFB_1. AFB_1-DNA adducts formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a biomarker for AFB_1 carcinogenic potential. Results are expressed as the mean ± SD. * significant difference ($P < 0.05$) from control value.



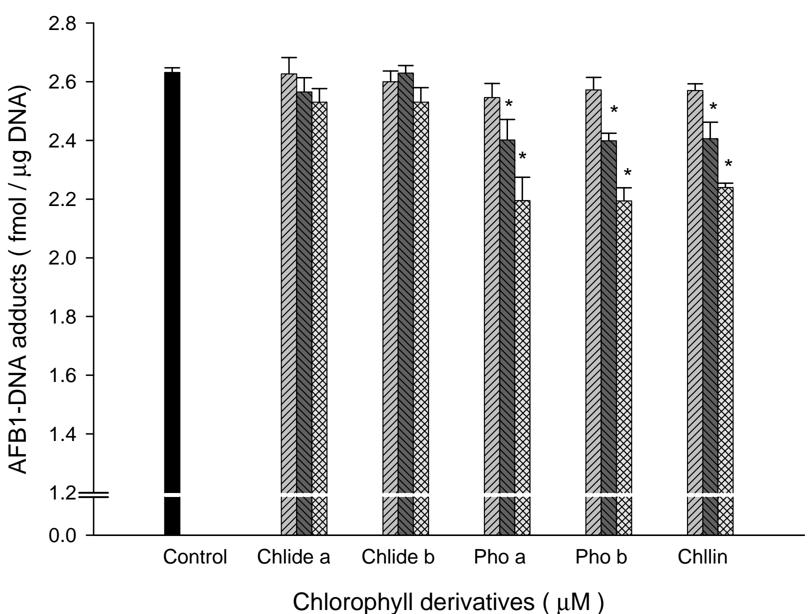
圖三十：Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後再給與 10 ng/mL AFB_1 所誘導 DNA 損傷的結果

Figure 30: Effect of pretreatment with chlorophyll derivatives before challenge with 10 ng/mL AFB_1. AFB_1-DNA adducts formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a biomarker for AFB_1 carcinogenic potential. Results are expressed as the mean ± SD. * significant difference ($P < 0.05$) from control value.



圖三十一：Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後 wash out 再給與 5 ng/mL AFB_1 所誘導 DNA 損傷的結果

Figure31: Effect of pretreatment with followed by wash-out of chlorophyll derivatives before challenge with 5 ng/mL AFB_1. AFB_1-DNA adducts formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a proxy for AFB_1 carcinogenic potential. Results are expressed as the mean ± SD. * significant difference ($P < 0.05$) from control value.



圖三十二：Hepa-1 細胞株在添加各種不同濃度的葉綠素水萃取物質後 wash out 再給與 10 ng/mL AFB_1 所誘導 DNA 損傷的結果

Figure 32: Effect of pretreatment with followed by wash-out of chlorophyll derivatives before challenge with 10 ng/mL AFB_1. AFB_1-DNA adduct formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a proxy for AFB_1 carcinogenic potential. Results are expressed as the mean ± SD. * significant difference ($P < 0.05$) from control value.

四、葉綠素水萃取物對於 Hepa-1 細胞株 GST 活性的影響

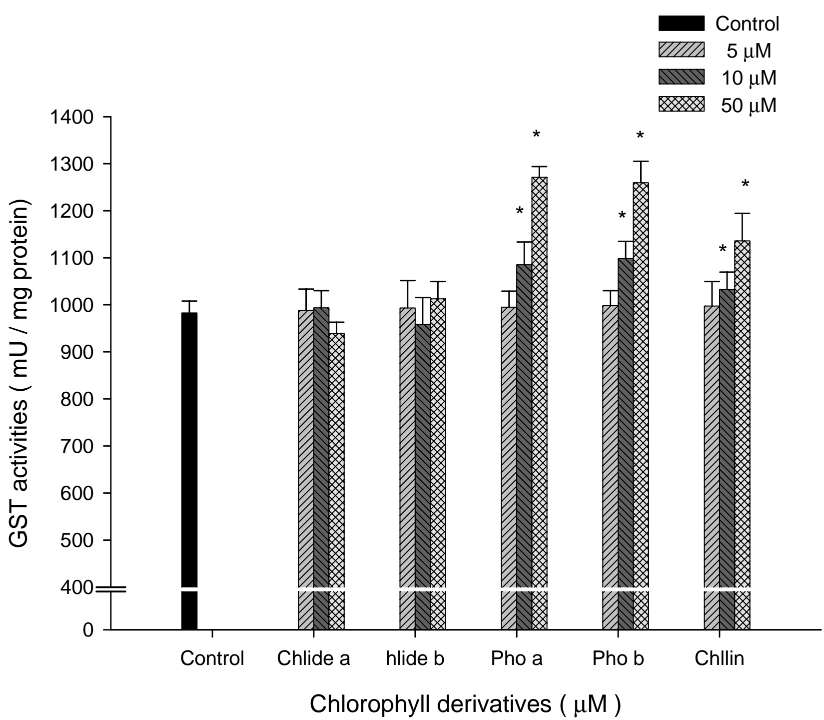
表九顯示 Hepa-1 細胞株在添加 AFB_1 後，GST 活性的結果。結果顯示 GST 的活性隨著 AFB_1 濃度的增加而降低。圖三十三與圖三十四　顯示 Hepa-1 細胞株在給與 AFB_1 前先加葉綠素水萃取物，其細胞內 GST 活性的結果。結果顯示所有葉綠素水萃取物中，GST 的活性隨著 Pho a 、b 與 Chlin 濃度的增加而增加，但是 Chlide 與對照組之間並無統計上的差異。這樣的結果可以部份解釋　明　何 Pho a 、b 在 washout 之後添加 AFB_1，仍對細胞具有保護的作用。

表九：AFB_1 對於 Hepa-1 細胞株 GST 活性的影響

Table 9: Effect of AFB_1 upon Hepa-1 GST activity[1].

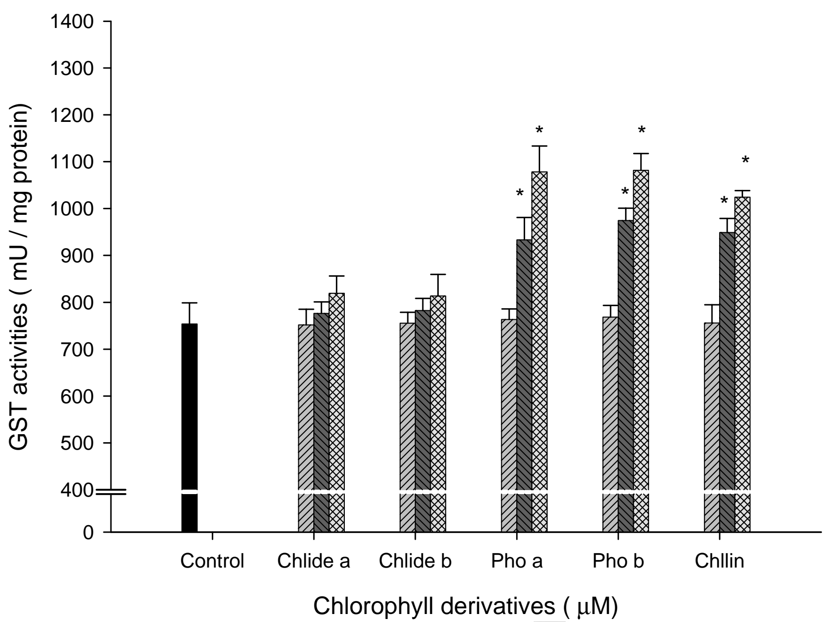
	GST activity
AFB_1 5 ng/mL	982.77±25.2
AFB_1 10 ng/mL	753.88±45.0
Control	1107.12±56.2

[1] mean±SD. Cells were treated for 48 h with 5,10 ng/mL AFB_1 to determine GST activity.



圖三十三：Hepa-1 細胞株在給與 5 ng/mL AFB_1AFB_1 前先加葉綠素水萃取物細胞內 GST 活性的結果

Figure 33: Effect of chlorophyll derivatives on GST activity in AFB_1-induced Hepa-1 cells. Cells were first treated with various derivatives for 48 h and then treated with 5 ng/mL AFB_1 for another 48 h. GST activity was evaluated using a commercial kit. Results are expressed as the mean ± SD. * significant difference ($P < 0.05$) from control value.



圖三十四： Hepa-1 細胞株在給與 10 ng/mL AFB_1AFB_1 前先加葉綠素水萃取物細胞內 GST 活性的結果

Figure 34: Effect of chlorophyll derivatives on GST activity in AFB_1-induced Hepa-1 cells. Cells were first treated with various derivatives for 48 h and then treated with 10 ng/mL AFB_1 for another 48 h. GST activity was evaluated using a commercial kit. Results are expressed as the mean ± SD. * significant difference ($P < 0.05$) from control value.

第三節 討論

一、植物性化學物質對抗黃麴毒素的探討

自從 1960 年代在英國發生了因餵食黃麴毒素的花生而導致大火雞暴　事件以來，類似事件還是不斷的在世界各國發生。例如在 2004 年自越南輸入日本的　被驗出含有超量的黃麴毒素； 2005 年自中國輸入日本的　麥粉也被驗出含有毒素。因此，各國　衛生組織都有　密的食品檢測，以避免這些毒素對人體造成肝腫瘤的發生。

根據國內　團法人肝病防治學術基金會的報導，許多危險因數是 B 型肝炎病毒帶原者日後發生肝癌的因素，這些因數包括 C 型肝炎病毒感染，抽菸、　　，攝取含有黃麴毒素的食物，以及新鮮蔬果攝取量較少等因素。研究顯示，每週攝食蔬果少於六　的 B 型肝炎帶原者，其罹患肝癌的危險性要比每週攝食蔬果六　以上的帶原者高四~五倍(肝炎防治會刊第三期)。由本研究的結果證實蔬果中的葉綠素，可以有效的抑制黃麴毒素誘導肝癌發生。

從細胞培養的研究上發現，　科植物中的　素(saponin, 10~50 μg/mL）對於 50 μg/mL 的黃麴毒素所誘導人類肝細胞株(HepG2)的研究上，具有保護 DNA 避免損傷的作用(Jun, et al., 2002）。推測　素是藉由抗氧化的功能來增加肝細胞中 GST 的活性以及 DNA 修補蛋白，進而減少 AFB_1-DNA adducts 的形成(Pool-Zobel, et al., 1998)。與

本研究相比較的時候發現，本實驗的第一部分利用淋巴球細胞所做的實驗，已經證實葉綠素的衍生物都具有抗氧化的作用；第二部分利用肝細胞株的研究亦證實：這些葉綠素衍生物亦可增加細胞中 GST 的活性，因此兩者在保護 DNA 損傷的部份具有相類似的結果。然而，過量的　素會破壞紅血球而引起溶血作用(Baumann, et al., 2000)，本研究利用葉綠素的衍生物並無此副作用的產生。

中西方常用的香草性植物對黃麴毒素也具有保護的效果，例如藥用植物中甘草(licorice root)的主要成分-甘草酸(glycyrrhizic acid)與常用做香料添加的紫草科(boraginaceae)成分-　　香酸(rosmarinic acid)；Chan 等學者 (2003)的研究中發現：人類肝細胞株(HepG2)同時與 0.5 µM 的黃麴毒素以及甘草酸(0.1µM-1mM)共同培養 30 分鐘之後，比較有無添加甘草酸的細胞內的酵素的表現有何不同，結果發現無添加甘草酸的細胞內的細胞色素 1A1 (CYP1A1)與 GST 的活性有顯著增加的情形，而細胞色素 1A2 (CYP1A2)則無顯著的差異，其中 GST 的表現也與本研究的結果相類似。相同的細胞株利用在　　香酸的研究上也發現：50µM 的　　香酸萃取物可以抑制 10 µM 的黃麴毒素對細胞所造成的毒性傷害(Renzulli, et al., 2004)。

在動物實驗的模式中，若以黃麴毒素誘導老鼠產生肝癌前細胞的病變，在飼料中添加十字花科蔬菜中萃取物- indole-3-carbinol (I3C,

0.5%)，十三週之後其體內肝臟解毒酵素如 CYP1A1、1A2、3A 以及 GST 的活性都有顯著的增加(Manson, et al., 1998)；此外每天餵食 300mg/kg 飼料的各種類胡蘿蔔素之後，再予以暴露在黃麴毒素 (2mg/kg body weight)的環境中，可以降低老鼠肝臟癌前病變的大小及數量，其中以 β-carotene、β-Apo-8'carotenal、astaxanthin 以及 canthaxanthin 四種類胡 蔔素的效果最好，其作用的機轉是這些類胡蘿蔔素不但可以降低 DNA 單骨斷裂，且可以將黃麴毒素 B_1 代謝成 M_1 的型態，進而減少對肝細胞的毒性傷害。

從流行病學的研究中發現：中國大 啟東地區的民眾，由於長期食用受到黃麴毒素所 染的食物，因此罹患肝癌的比例一直居高不下，在進行人體研究發現：當暴露黃麴毒素高危險 的受試者每日給予 5 毫克的 Oltipraz (圖三十五)藥物八週之後，血液中的 GST 活性(Wang, et al., 1999)與尿液中 AFB_1-mercapturic acid 的含量 (Jacobson, et al., 1997)都明顯的比對照組增加，顯示 Oltipraz 藉由提高 GST 的活性，進而催化 AFB_1-2,3- epoxide 與 GSH 結合而形成親水較高的產物(AFB_1-mercapturic acid)，藉此達到對抗黃麴毒素解毒的作用 (Bolton, et al., 1993)。此外，十字花科蔬菜中的 dithiolethiones 衍生物，因為其結構與 Oltipraz 相類似，因此在體內對抗黃麴毒素也具有相同的作用(Roebuck, et al., 2003)。

圖三十五：Oltipraz 的化學結構

Fig 35: The chemical structure of oltipraz

二、葉綠素對黃麴毒素誘導基因突變的保護探討

有關葉綠素對抗黃麴毒素基因毒性的保護，過去研究最多的就是銅鈉葉綠素(圖十二）(Dashwood, et al., 1998）；近年來，Enger 等學者(2001)發表，針對肝癌發生率較高的中國大　　　　所進行的人體試驗研究，發現每天給予健康的受試者 300 毫克的 Chlin，四個月之後測量尿液中黃麴毒素與 DNA 結合的產物，發現較原先未攝取前減少了 55%，證實口服葉綠素製品可以降低黃麴毒素對肝臟細胞的傷害。然而銅鈉葉綠素雖然屬於食品級的添加物，但是它並非存在於天然的蔬菜水果之中，本研究的葉綠素水萃取物完全來自於台灣新鮮菠菜，藉由中央研究院植物所楊棋明博士成功的萃取、分離而得，因此其結果更可證明天然的蔬果對於環境中的毒性物質具有不同程度的的保護作用。

葉綠素對於黃麴毒素誘發肝癌保護效果的生理機轉，可以從下列二方面來解釋。

(一)、利用分子捕捉(molecular trapping)的效應與黃麴毒素結合

Porphyrin 的化學結構，可藉由分子平面結構的 水作用力(hydrophobic interactions)的作用與致突變物質形成複合體，因而降低了致突變物質與細胞中 DNA 結合而造成的突變傷害(Hayatsu, 1992；Dashwood and Liew, 1992)。換言之，葉綠素是藉由其分子結構的特性，利用分子捕捉的效應，與黃麴毒素結合， 斷了黃麴毒素對肝細胞中 DNA 的基因突變。本研究利用細胞培養的實驗，讓葉綠素萃取物與細胞共同培養之後，再加入黃麴毒素，則細胞中 DNA 與黃麴毒素結合產物明顯的降低，顯然葉綠素在直接接觸黃麴毒素的情況下，發揮了分子捕捉的效應。

(二)、提高細胞解毒酵素酵素活性， 斷了黃麴毒素的活化

Fahey 等學者(2005)利用體外實驗發現，Chlin 可抑制小鼠肝癌細胞和誘導解毒酵素 II NAD(P)H:Quinone Oxidoreductase 1（NQO1）。此外，針對葉綠素與 GST 的研究上，以 7,12-dimethylbenz [a]anthracene (DMBA)誘導小鼠 形成乳狀瘤的前後，若給予口服 Chlin 則會明顯的增加肝與 中 GST 的活性，同時也減少產生癌化的病變產生(Singh, et al., 1996)。此結果在 GST 表現上與本研究中的 Chlin 和 Pho a, b 有相同的 ；但 Simonich 等學者(2007) 以大鼠體內實驗進行的研究上發現，若給予天然葉綠素後，對於預防黃麴毒素 B_1 的毒性

上，可以藉由「黃麴毒素 B_1-葉綠素複合物」的形成而降低 AFB_1 DNA adducts 的生成；但卻不影響解毒酵素 NQO1 以及 GST 的活性。此結果在 GST 表現上與本研究中的 Pho a, b 是相同的。

上述學者利用葉綠素所做出來的結果 異，推測與葉綠素衍生物的結構不同有關。在本實驗中發現，Chlin 和 Pho a, b 具有明顯的增加肝細胞中 GST 的活性，而 Chlide a, b 卻無法增加肝細胞中 GST 的活性，推測是 Hepa-1 細胞株對不同的葉綠素衍生物具有不同的通透性所致。與正常細胞相比，Hepa-1 細胞株屬於小鼠肝腫瘤細胞，研究指出，腫瘤細胞容易吸收 Pho a (Hayashi, et al., 1985；Chernomorsky, et al., 1999；Hajri, et al., 2002)，濃縮 入粒線體中(Tang, et al., 2006；Kim, et al., 2004) 。此外，AFB_1 與 Pho 出細胞的能力都是藉由與 ATP 結合蛋白（ATP-binding cassette，例如乳癌 抗蛋白 breast cancer resistance protein）結合的方式被 出細胞(Herwaarden, et al., 2006；Jonker, et al., 2002)。過去的研究中都認為 Hepa-1 細胞株中能表現 AFB_1 的基因毒性(Karenlampi, 1987)，表示 AFB_1 穩定的存在細胞內，因此推測 Hepa-1 細胞株有較低 ATP 結合蛋白，而減少黃麴毒素被 出去的機會；同時也因為具有較低的 ATP 結合蛋白，因此降低細胞中脫鎂脫植醇葉綠素 a 被細胞 出去的機會，進而留在細胞中，一方面可與 AFB_1 結合而降低對 DNA 的損傷，另一方面活化 GST 的表現

而增加解毒酵素的功能。惟研究葉綠素對細胞間通透性的文獻不多，有關於細胞中 ATP 結合蛋白與其他葉綠素的機轉仍需要更多的研究來證實。

在本研究中，當細胞在加入黃麴毒素之前，若將原本存在細胞培養液中的葉綠素去除，其結果發現 Pho 還是具有少量保護的效果，證實這些少量的保護效果是來自於 Pho 在先前的與細胞培養時就進入細胞並留在細胞中，藉由活化 GST 的活性而降低黃麴毒素 B_1 的毒性，來減少 AFB_1 DNA adducts 的生成。

第五章　結論

葉綠素水萃取的代謝產物可以降低對過氧化氫所誘導淋巴球細胞 DNA 的損傷，其中在低濃度的氧化傷害之下，無論是脫植醇葉綠素 a、b ，脫鎂脫植醇葉綠素 a、b 或是銅鈉葉綠素對淋巴球 DNA 的氧化損傷都具有保護的效果；在高濃度的氧化傷害之下，僅脫鎂脫植醇葉綠素 a、b 具有保護 DNA 損傷的功效。從 in vitro 的抗氧化實驗中也證實以脫鎂脫植醇葉綠素的抗氧化能力最強，顯示這些植物性化學物質可以發揮自由基捕捉的效應來降低氧化傷害；從這些化合物的比較可推測脫鎂脫植醇葉綠素同時亦具有螯合亞鐵離子的能力而發揮最好的抗氧化作用。

此外，對於黃麴毒素所誘導肝細胞 DNA 損傷的研究中，發現葉綠素水萃取物可降低 AFB_1 DNA adduct 的生成，其中脫鎂葉綠素可與黃麴毒素 B_1 結合，減少黃麴毒素 B_1 對肝細胞 DNA 的損傷；銅鈉葉綠素和脫鎂脫植醇葉綠素 a, b 則透過與黃麴毒素 B_1 結合之外，還可藉由活化 GST 的活性而增加肝細胞解毒酵素的功能，因此抑制 AFB_1 DNA adduct 生成的作用較明顯。

藉由本研究證實部分葉綠素衍生物之保護作用的機轉，並　明飲食中攝取富含葉綠素的蔬果，可預防癌症之發生。

第六章 參考文獻

團法人肝炎防治學術基金會(1997) 保肝十大　則。肝炎防治會刊第三期。

拱玉郎 (1997) 天然抗氧化劑發　近況。食品工業月刊　29：29-37。

葉　　(2003) 哺乳動物對葉綠素新陳代謝之研究。中國文化大學生物科技研究所碩士論文。

錢明賽 (1998) 蔬果中之抗氧化物質。食品工業月刊 30: 21-34。

Almela L, Fernandez-Lopez JA and Roca MJ (2000) High-performance liquid chromatographic screening of chlorophyll derivatives produced during fruit storage. J.Chromatogragphy A 870: 483-489.

Autrup H, Seremet T and Wakhisi J (1987) Aflatoxin exposure measured by urinary excretion of aflatoxin B1-guanine adduct and hepatitis B virus infection in areas with different liver cancer incidence in Kenya. Cancer Res 47: 3430-3433.

Baxter JH (1968) Absorption of chlorophyll phyto in normal man and in patients with Refsums disease. J Lipid Res 9: 636-641.

Baumann E, Stoya G, Völkner A, Richter W, Lemke C and Linss W (2000) Hemolysis of human erythrocytes with saponin affects the membrane structure. Acta Histochem 102: 21-35

Bishayee A, Sarkar A and Chatterjee M (1995) Hepatoprotective activity of carrot against carbon tetrachloride intoxication in mouse liver. J Ethnopharmacolo 47: 69-74.

Block G, Patterson B and Subar A (1992) Fruits, vegetables and cancer prevention: A review of the epidemiology evidence. Nutr Cancer 18: 1-29.

Bolton MG, Munoz A, Jacobson LP, Groopman JD, Maxuitenko YY, Roebuck BD and Kensler TW (1993) Transient intervention with oltipraz protects against aflatoxin-induced hepatic tumorigenesis. Cancer Res 53:3499-3504.

Bonorden WR and Pariza MW (1994) Antioxidant nutrients and protection from free radicals, In: Nutr. Toxicol, Kostsonis FN, Mackey M and Hjelle J ed. Raven press. New York. 19-48.

Borg DC, Fajer J, Felton RH and Dolphin D (1970) The π-Cation Radical of Chlorophyll a. PNAS 67: 813-820.

Bortlik K, Peisker C and Matile P (1990) A novel type of chlorophyll catabolite in senescence Barley leaves. J Plant Physiol 136: 161-165.

Briges AB, Scott NA, Pringle TH, McNeill GPB and Elch JJ (1992) Relationship between the extent of coronary artery disease and

indicators of free radical activity. Clin Cardiol 15: 169-174.

Bulter WH and Barnes TM (1963) Toxic effects of groundnut meal containing aflatoxin to rats and guinea pigs. Br J Cancer 17: 699-710.

Busby WF and Wogan GN Chemical Carcinogen. Vol. 2-second edition

Carnaghan RBA (1965) Hepatic tumors in duck fed a low level of toxic groundnut meal. Nature 208: 308

Chan HT, Chan C and Ho JW (2003) Inhibition of glycyrrhizic acid on aflatoxin B1-induced cytotoxicity in hepatoma cells. Toxicology 188: 211–217.

Chen CJ, Chang YC and Tasi SF (1988) Epidemiological studies of multiple risk factors in hepatocellular carcinoma in Taiwan. In: Zuckerman AJ (ed.) Viral Hepatitis and Liver Diseases. Alan R. Liss Inc New York 723-725.

Chernomorsky S, Segelman A and Poretz RD (1999) Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth. Teratog Carcinog Mutagen 19: 313-322.

Coles B, Meyer DJ, Ketterer B, Stanton CA and Garner RC (1985) Studies on the detoxication of microsomally-activated aflatoxin B_1 by glutathione and glutathione S-transferase in vitro. Carcinogenesis 6:

693-697.

Cory AH, Owen TC, Barltrop JA and Cory JG. (1991) Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun.3:207-212.

Dashwood R and Liew C (1992) Chlorophyllin-enhanced excretion of urinary and fecal mutagens in rats given 2-amino-3-methylimidazo [4,5-f] quinoline. Environ Mol Mutagen 20: 199-205.

Dashwood R, Negishi T, Hayatsu H, Breinholt V, Hendricks J, and Bailey G (1998) Chemopreventive properties of chlorophylls towards aflatoxin B_1: a review of the antimutagenicity and anticarcinogenicity data in rainbow trout. Mutat Res 399:245-253.

Day BJ, Batinic Haberle I and Crapo JD (1999) Metalloporphyrins and potent inhibitors of lipid peroxidation. Free Radic Biol Med 26: 730-736.

Day BJ, Fridovich I and Crapo JD (1997) Manganic porphyrins possess catalase activity and protect endothelial cell against hydrogen peroxide-mediated injury.Arch Biochem Biophys 347: 256-262.

Dreher D and Junod AF (1996): Role of oxygen free radicals in cancer development. Eur J Cancer 32A: 30-38.

Egner PA, Wang JB, Zhu YR, Zhang BC, Wu Y, Zhang QN, Qian GS, Kuang SY, Gange SJ, Jacobson LP, Helzlsouer KJ, Bailey GS, Groopman JD and Kensler TW (2001) Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. Proc Natl Acad Sci U S A 98: 14601-14606.

Endo Y, Usuki R and Kaneda T (1985) Antioxidant effects of chlorophyll and pheophytin on the autoxidation of oil in the dark .II. The mechanism of antioxidative action of chlorophyll. J Am Oil Chem Soc 62: 1387-1390.

Fahey JW, Stephenson KK, Dinkova-Kostova AT, Egner PA, Kensler TW and Talalay P (2005) Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes. Carcinogenesis 26: 1247-1255.

Ferruzzi MG, Failla ML, Schwartz SJ (2001) Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and Caco-2 human cell model. J Agric Food Chem 49: 2082-2089.

Forster PL, Eisenstadt E, Miller JH (1983) Base substitution mutation induced by metabolically activated aflatoxin B1. Proc Natl Acad Sci U.

S. A. 80: 2695-2698.

Foyer CH and Fletcher JM (2001) Plant antioxidants: colour me healthy.Biologist 48: 115-120.

Frenzilli G, Bosco E and Barale R (2000) Validation of single cell gel assay in human leukocytes with 18 reference compounds. Mutat Res 468:93-108.

Gurtoo HL (1980) Genetic expression of aflatoxin B1 metabolism: effect of 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzen-zo-p-dioxin on metabolism of aflatoxin B1 and B2 by various inbred strains of mice. Mol Pharmacol 18: 296-303.

Gutteridge JMC and Halliwell B (1990) Reoxygenation injury and antioxidant protection: a tale of two paradoxes. Arch Biochem Biophys 283: 223-226.

Hagen U (1989) Biochemical aspects of radiation biology. Experientia 45:7-12.

Hajri A, Wack S, Meyer C, Smith MK, Leberquier C, Kedinger M and Aprahamian M (2002) In vitro and in vivo efficacy of photofrin and pheophorbide a, a bacteriochlorin, in photodynamic therapy of colonic cancer cells. Photochem Photobiol 75: 140-148.

Halliwell B (1998) Can oxidative DNA damage be used as a biomarker of cancer risk in human? Free Radic Res. 29: 469-486.

Halliwell B (2000) Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come[]? Am J Clin Nutr 72: 1082-1087.

Halliwell B and Gutteridge JMC (1999) Free radical in biology and medicine. 3th ed., Clarendon press, Oxford. 284-313.

Hapuarachchi JR, Chalmers AH, Winefield AH and Blake-Mortimer JS. (2003) Changes in clinically relevant metabolites with psychological stress parameters. Behav Med 29: 52-59.

Hayatsu H (1992) Formation of mutagen-porphyrin complex as a mechanism in antimutagenesis, Sixth International Conference on Environmental Mutagens. (Abs). 184:66c

Hayashi H, Nakajima S, Samejima N (1985) Basic study for cancer therapy with porphyrin derivatives and pheophorbide derivatives, Hokkaido Igaku Zasshi 60: 391-403.

Hayashi M, Yamazoe H, Yamaguchi Y and Kunitomo M (1992) Effects of green tea extract on galactosamine-induced hepatic injury in rats. Nippon Yakurigaku Zasshi 100: 391-399.

Helbock HJ, Backman KB and Shigenaga MK (1998) DNA oxidation

matters:the HPLC- clectrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. Proc Natl Acad Sci U.S.A. 95: 288-293.

Herwaarden AE, Wagenaar E, Karnekamp B, Merino G, Jonker JW, Schinkel AH (2006) Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. Carcinogenesis 27: 123-130.

Hsieh LL and Hsieh TT (1993) Detection of aflatoxin B1-DNA adducts in human placenta and cord blood. Cancer Res 53: 1278-1280.

Jacobson LP, Zhang BC, Zhu YR, Wang JB, Wu Y, Zhang QN, Yu LY, Qian GS, Kuang SY, Li YF, Fang X, Zarba A, Chen B, Enger C, Davidson NE, Gorman MB, Gordon GB, Prochaska HJ, Egner PA, Groopman JD, Munoz A, Helzlsouer KJ and Kensler TW (1997) Oltipraz chemoprevention trial in Qidong, People's Republic of China: study design and clinical outcomes. Cancer Epidemiol Biomarkers Prev 6: 257-265.

Janeric S, Pero RW, Markowitz MM, George R, Miller DG and Beattie EJ (1990) Isoenzyme(s) of glutathione transferase(class Mu) as a marker

for the susceptibility to lung cancer:a follow up study. Carcinogenesis 11:33-36.

Janero DR (1990) Malondialdehyde and thiobarbituric acid-reacitvity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radi Biol Med 9: 515-540.

Jayaraj RK, Terner J, Gold A, Roberts DA, Austin RN, Mandon D,Weiss R, Bill E and Trautwein AX (1996) Influence of meso Substituents on Electronic States of Oxoferryl Porphyrin π-Cation Inorg Chem 35:1632-1640.

Jayashree T and Subramanyam C (2000) Oxidative stress as a prerequisite for aflatoxin production by Aspergillus parasiticus. Free Radic Biol Med 29: 981-985.

Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH and Schinkel AH (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc Natl. Acad Sci U. S. A. 99: 15649-15654.

Jun HS, Kim SE and Sung MK (2002) Protective effect of soybean saponins and major antioxidants against aflatoxin b1-induced

mutagencity and DNA-adducts formation. J Med Food 5: 235-240.

Kadiiska MB, Mason RP, Dreher KL, Costa DL and Ghio AJ (1997) In vivo evidence of free radical formation in the rat lung after exposure to an emission source air pollution particle. Chem Res Toxicol 10: 1104-1108.

Kamat JP, Boloor KK, and Devasagayam TPA (2000) Chlorophyllin as an effective antioxidant against membrane damage in vitro and ex vivo. Biochim. Biophys. Acta 1487: 113-127.

Karenlampi SO (1987) Mechanism of cytotoxicity of aflatoxin B1: role of cytochrome P1-450. Biochem Biophys Res Commun. 145: 854-60.

Kasai H, Hayami H, Yamaizumi Z, Saito H and Nishimura S(1984): Detection and identification of mutagens and carcinogens as their adducts with guanosine derivatives. Nucleic Acids Res 12: 2127-2136.

Kim CS, Lee CH, Lee PH and Han S (2004) Inactivation of mitochondrial electron transport by photosensitization of a pheophorbide a derivative. Mol Cells 17: 347-352.

Koser PL, Faletto MB, Maccubbin AE and Gurtoo HL (1988) The genetics of aflatoxin B1 metabolism: association of the induction of aflatoxin B1-4-hydroxylase with the transcriptional activation of cytochrome P3-450 gene. J Biol Chem 263: 12584-12595.

Kumar SS, Devasagayam TP, Bhushan B and Verma NC (2001) Scavenging of reactive oxygen species by chlorophyllin: an ESR study. Free Radic Res 35: 563-574.

Kumar SS, Chaubey RC, Devasagayam TPA, Priyadarsini KI and Chauhan PS (1999) Inhibition of radiation-induced DNA damage in plasnid pBR 32 by chlorophyllin and possible mechanism(s) of action. Mutat Res 425: 71-79.

Lai CN (1979) Chlorophyll: the active factor in wheat sprout extract inhibiting the metabolic activation of carcinogens in vitro. Nutr Cancer 1: 27-30.

Lai CN, Butler MA and Matney TS (1980) Antimutagenic activities of common vegetables and their chlorophyll content. Mutat Res 77: 245-250.

Lampe JW, Chen C, Li S, Prunty J, Grate MT, Meehan DE, Barale KV, Dightman DA, Feng Z and Potter JD (2000) Modulation of human glutathione S-transferases by botanically defined vegetable diets. Cancer Epidemiol Biomarkers Prev 9: 787-793.

Lanfer-Marquez MU, Barros RMC and Sinnecker P (2005) Antioxidant activity of chlorophylls and their derivatives. Food Res Intern 38:

885-891.

Longo FR, Brown EM and Quimby DJ (1973) Kinetic studies on metal chelation by porphyrins. Ann N Y Acad Sci 206: 420-442.

L'opez-Ayerra B, Antonia MM and Garcia-Carmona F. (1998) Lipid peroxidation and chlorophyll levels in spinach during refrigerated storage and after industrial processing. Food Chem 61: 113-118.

Lunec J ESCODD (1998) European Standards Committee of Oxidative DNA Damage. Free Radic Res 2: 601-608.

Machlin LJ and Bendich A (1987) Free radical tissue damage: protective role of antioxidant nutrients. FASEB J.1: 441.

Manson MM, Hudson EA, Ball HW, Barrett MC, Clark HL, Judah DJ, Verschoyle RD and Neal GE (1998) Chemoprevention of aflatoxin B1-induced carcinogenesis by indole-3- carbinol in rat liver--predicting the outcome using early biomarkers. Carcinogenesis 19:1829-1836

Massey V (1994) Activation of molecular oxygen by flavins and flavoproteins. J Biol Chem 269: 22459-22462.

McDermott JH (2000) Antioxidant nutrient: current dietary recommendations and research update. J Am Pharm Assoc 40:

785-799

McElroy DC, Mauzerall CD and Feher G (1974) Characterization of primary reactants in bacterial photosynthesis II. Kinetic studies of the light-induced signal (g = 2.0026) and the optical absorbance changes at cryogenic temperatures. Biochim. Biophys. Acta 333: 261-278.

Milano J and Day BJ (1999) : catalytic antioxidant metalloporphyrin blocks hydrogen peroxide-induced mitochondrial DNA damage. Nucleic Acids Research 28: 968-973.

Negishi T, Rai H and Hayatsu H (1997) Antigenotoxic activity of natural chlorophylls. Mutat Res 376: 97-100.

Nishikawa M, Kakemizu N, Ito T, Kudo M, Kaneo T, Suzuki M, Udaka N, Ikeda H and Okubo T (1999) Superoxide mediates cigarette smoke-induced infiltration of neutrophils into airways through nuclear factor-kappa B activation and IL-8 mRNA expression in guinea pigs in vivo. Am J Respir Cell Mol Biol 20: 189-198.

Okamoto K, Toyokuni S, Kim WJ, Ogawa O, Kakehi Y, Arao S, Hiai H and Yoshida O. (1996) Overexpression of human mutt homologue gene messenger RNA in renal-cell carcinoma: evidence of persistent oxidative stress in cancer. Int J Cancer 65:

437-441.

Ong T, Whong WZ, Stewart J and Brockman HE (1989) Comparative antimutagenicity of five compounds against five mutagenic complex mixtures in salmonella typhimurium atrain TA 98. Mutat Res 222: 19-25.

Olive PL, Banath JP and Durand RE (1990) Heterogeneity in radiation induced DNA damage and repair in tumor and normal cells measured using the "Comet assay". Radiat Res 122: 86-94.

Ostling O and Johanson KJ (1984) Microelectrophoretic study of radiation induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 123: 291-298.

Pasternack RF, Banth A, Pasternack JM and Johnson CS (1981) Catalysis of the disproportionation of superoxide by metalloporphyrins. J Inorg Biochem 15: 261-267.

Peers FG, Cilman GA and Linsell CA (1976) Dietary aflatoxins and human liver cancer, a study in swaziland. Int J Cancer 17: 167-176.

Pool-Zobel BL, Bub A, Liegibel UM, lishaut ST and rechkemmer G (1998) Mechanisms by which vegetable consumption reduces genetic damage in humans. Cancer Epidemiol Biomarkers Prev. 7:891-899.

Prochasks HJ, Delong MJ and Talalay P (1985) On the mechanisms of induction of cancer-protective enzyme: A unifying proposal. Proc Natl Acad Sci U.S.A. 82: 8232-8236.

Ravikanth M, Misra A, Reddy D and Chandrashekar TK (1994) π-Cation radicals of Iron(III) derivatives of deformed porphyrins. J Chem Soc Dalton Trans 491-495.

Ravikanth M, Misra A, Chandrashekar TK, Sathiah S and Bist HD (1994) π-Cation radicals of copper(II) derivatives of short chain basket handle porphyrins. Inorg. Chem 33: 392-394.

Renner M W, Fajer J (2001) Oxidative chemistry of nickel porphyrins. J Biol Inorg Chem 6:823-830.

Rensburg S.J, Cook-Mozaffari P and Van Schalkwyk DJ (1985) Hepatocellular carcinoma and dietary aflatoxin in Moxambique and Transkei. Br J Cancer 51: 713-726.

Renzulli C, Galvano F, Pierdomenico L, Speroni E, Guerra MC (2004) Effects of rosmarinic acid against aflatoxin B1 and ochratoxin-A-induced cell damage in a human hepatoma cell line (Hep G2).J Appl Toxicol 24:289-296.

Roebuck BD, Curphey JT, Li Y, Baumgartner KJ, Bodreddigari S, Yan J,

Gange SJ, Kensler TW and Sutter TR (2003) Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. Carcinogenesis 24: 1919-1928.

Sargeant K, Sheridan A, Kelly JO and Carnaghan RBA (1961) Toxicity associated with certain samples of ground nuts. Nature. 192: 1096-1097.

Sarker AH, Watanabe S, Seki S, Akiyama T and Okada S (1995) Oxygen radical-induced single –strand Dna Breaks and repair of the damage in a cell-free system. Mutat Res 337: 85-95.

Sato TY, Suzuki YO and Ishikawa T (1984) Desmutagenic substance in water extract of grass-wrack pondweed. Mutat Res 129: 33-38.

Sato M, Imai K and Murata T (1977) Effect of sodium copper chlorophyllin on lipid peroxidation: I.Effect on lipid peroxidation in rat liver homogenates in the presence of both Fe2+ and L-ascorbic acid. Yakugaku Zasshi 97: 268-273.

Scheidt WR, Brancatobuentello KE, Song HS, Reddy V and Cheng B (1996) Metalloporphyrin Mixed-Valence Pi-Cation Radicals-Solid-State Structures. Inorg Chem 35:7500-7507.

Schuller P (1976) A review of sampling plans and collaboratively studied

methods of analysis for aflatoxins. J AOAC Int 6:1315.

Shen HM, Ong CN, Shi CY (1995) Involvement of reactive oxygen species in aflatoxin B1-indeced cell injury in cultured rat hepatocytes. Toxicology 99: 115-123

Shen HM, Shi CY, Shen Y. and Ong CN (1996) Detection of elevated reactive oxygen species level in cultured rat hepatocyte treated with aflatoxin B1. Free Radic Biol Med 24: 139-146.

Shimada K, Fujikawa K, Yahara K and Nakamura T (1992) Antioxidative properties of xanthone on the auto oxidation of soybean in cylcodextrin emulsion. J Agri Food Chem 40: 945-948.

Shimada T and Guengerich FP (1989) Evidence for cytochrome P450NF, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. Proc natl Acad Sci U.S.A. 86: 462-465.

Sieber SM, Correa Pelayo, Dalgard DW (1979) Introduction of osteogenic sarcomas and tumors of the hepatobiliary system in nonhuman primates with aflatoxin B_1. Cancer Res 39: 4545-4554.

Sies H (1986) Biochemistry of oxidative stress. Agnew Chem Int Ed Engl 25: 1058-1071.

Simonich MT, Egner PA, Roebuck BD, Orner GA, Jubert C, Pereira C, Groopman JD, Kensler TW, Dashwood RH, Williams DE and Bailey GS (2007) Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat. Carcinogenesis 28: 1294-1302.

Sinclair JF, Sinclair PR, Healey JF, Smith EL, and Bonkowsky HL (1982) Decrease in hepatic cytochrome P-450 by cobalt. Evidence for a role of cobalt protoporphyrin. Biochem J 204: 103-109.

Singh A, Singh SP and Bamezai R (1996) Modulatory influence of chlorophyllin on the mouse skin papillomagenesis and xenobiotic detoxication system. Carcinogenesis 17: 1459-1463.

Singh NP, McCoy MT, Tice RR and Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175: 184-191.

Slobodan VJ and Michael GS (2000) Antioxidants in nutrition. Annal of the New York Academy of Sciences 899: 326-334

Smith LW (1944) Chlorophyll: experimental study of its water- soluble derivatives. J Am Med Soc 207: 647-654.

Swinson DH, Lin JK, Miller EC and Miller JA (1977) Aflatoxin B1-2,3-oxide as a probable intermediate in the covalent of aflatoxin

B1 and B2 to rat DNA and ribosomal RNA in vivo. Cancer Res 37: 172-181.

Szabo C, Day BJ and Salzman AL (1996) Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger.FEBS Lett 381: 82-86.

Szeto HH (2006) Mitochondria-Targeted Peptide Antioxidants: Novel Neuroprotective Agents. The AAPS Journal 8: E521-E531.

Tang PM, Chan JY, Au SW, Kong SK, Tsui SK, Waye MM, Mak TC, Fong WP and Fung KP (2006) Pheophorbide a, an active compound isolated from Scutellaria barbata, possesses photodynamic activities by inducing apoptosis in human hepatocellular carcinoma. Cancer Biol Ther 5: 1111-1116.

Teel RW and Huynh H (1998) Modulation by Photochemicals of cytochrome P450-linked enzyme activity. Cancer Lett 133: 135-141.

Trebitsh T, Goldschmidt EE, Riov J (1993) Ethylene induces de novo synthesis of chlorophyllase, a chlorophyll degrading enzyme, in citrus fruit peel. Proc Natl Acad Sci U.S.A. 90: 9441-9445.

Wang JS, Shen X, He X, Zhu YR, Zhang BC, Wang JB, Qian GS, Kuang SY, Zarba A, Egner PA, Jacobson LP, Muñoz A, Helzlsouer KJ, Groopman JD and Kensler TW (1999) Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. J Natl Cancer Inst 91:347-354.

Warner JR, Joginder N and Ong T. (1991) Antimutagenicity studies of chlorophyllin using the Salmonella arabinose resistant assay system. Mutat Res 262: 25-30.

Wei H, Zhang X, Zhao JF, Wang ZY, Bickers D and Lebwohl M (1999) Scanvenging of hydrogen peroxide and inhibition of ultraviolet light-induced oxidative DNA damage by aqueous extracts from green tea and black tea. Free Radic Biol Med 26: 1427-1435.

Weingarten M and Payson B (1951) Deodorization of colostomies with chlorophyllin. Rev Gastroenterol 18: 602-604.

Whong W, Stewart J, Brockman HE and Ong T (1988) Comparative antimutagenicity of chlorophyllin and five other agents against aflatoxin B1-induced recersion in salmonella typhimurium atrian TA 98. Teratog Carcinog Mutagen 8: 215-224.

Wogan GN and Newberne PM (1967) Dose-response characteristics of

aflatoxin B1 carcinogenesis in the rat. Cancer Res 31: 1936-1942

Wolf B and Christa M (2002) Chemistry of the antioxidant effect of polyphenols. Ann N Y Acad Sci 957: 57-69.

Yeh FS, Yu MC and Mo CC (1989) Hepatitis B virus, aflatoxins and hepatocellular carcinoma in southern Guangxi, China. Cancer Res 49: 2506-2509.

Young AL and Lowe GM (2001) Antioxidant and prooxidant properties of carotenoids. Arch Biochem Biophys 385: 20-27.

Young RW and Bergei JS (1980) Use of chlorophyllin in the care of geriatric patients. J Am Geriatr Soc 28: 46-47.

Yun CH, Jeong HG, Jhoun JW and Guengerich FP (1995) Non-specific inhibition of cytochrome P450 activities by chlorophyllin in human and rat liver microsomes. Carcinogenesis 16: 1437-1440.

Zuber R, Modriansky M, Dvorak Z, Rohovsky P, Ulrichova J, Simanek V and Anzenbacher P (2002) Effect of silybin and its congeners on human liver microsomal cytochrome P450 activities. Phytother Res 16: 632-638.

第七章 已發表之期刊論文　　5-　　）

<u>Hsu CY</u>, Yang CM, Chen CM, Chao PY and Hu SP (2005) Effects of chlorophyll-related compounds on hydrogen peroxide induced DNA damage within human lymphocytes. J Agric Food Chem 53: 2746-2750.

Chen CM, Li SC, Lin YL, **<u>Hsu CY</u>**, Shieh MJ and Liu JF.(2005) Consumption of purple sweet potato leaves modulates human immune response: T-lymphocyte functions, lytic activity of natural killer cell and antibody production. World J Gastroenterol 11:5777-5781.

<u>Hsu CY</u>, Chen HH, Chao PY, Chen CM, Hsieh LL and Hu SP (2008) Naturally occurring chlorophyll derivatives inhibit aflatoxin B_1-DNA adduct formation in hepatoma cells. Mutat Res 657: 98-104.

Chen CM, Lin YL, Chen CY, **<u>Hsu CY</u>**, Shieh MJ and Liu JF (2008) Consumption of purple sweet potato leaves decreases lipid peroxidation and DNA damage in humans. Asia Pac J Clin Nutr 17:408-414.

2746 *J. Agric. Food Chem.* **2005,** *53,* 2746–2750

Effects of Chlorophyll-Related Compounds on Hydrogen Peroxide Induced DNA Damage within Human Lymphocytes

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Chlorophylls (Chl's) are the most abundant natural plant pigments. Four chlorophyll-related compounds (CRCs), including chlorophyllide *a* and *b* (Chlide *a* and *b*) and pheophorbide *a* and *b* (Pho *a* and *b*), were investigated for their antioxidative capacities to protect human lymphocyte DNA from hydrogen peroxide (H_2O_2) induced strand breaks and oxidative damage ex vivo. Lymphocytes exposed to H_2O_2 at concentrations of 10 and 50 μM revealed an increased frequency of DNA single-strand breaks (ssb's; as measured by the comet assay) and also an increased level of oxidized nucleoside (as measured by 8-hydroxydeoxyguanosine, 8-OHdG). All Chl's reduced the level of DNA ssb's and 8-OHdG within human lymphocytes following exposure to 10 μM H_2O_2. Only Pho *a* and *b* were able to decrease DNA ssb's and 8-OHdG following treatment of lymphocytes with 50 μM H_2O_2, in a concentration-dependent fashion. It was demonstrated herein that Pho *a* and *b* were more antioxidative than others. We applied DPPH free-radical scavenge assays in vitro, and got similar results. Pho *a* and *b* had higher ability in scavenging capacities than others. We conclude that water-extract Chl's are able to enhance the ability of human lymphocytes to resist H_2O_2-induced oxidative damage, especially for Pho *a* and *b*.

KEYWORDS: **Chlorophyll; hydrogen peroxide; DNA damage; comet assay; 8-hydroxydeoxyguanosine (8-OHdG)**

INTRODUCTION

Free radicals and oxygen radicals are constantly generated in vivo and cause oxidative damage to DNA that is probably a significant contributor to cancer. Epidemiological studies have shown an inverse relationship between the consumption of vegetables and the incidence of human cancer (*1*). For most cancer sites, individuals who reflect a low fruit and vegetable intake experience about twice the risk of cancer compared with those who feature a high intake of fruit and vegetables (*2*).

Chlorophylls (Chl's) are the most abundant and widely distributed green pigments found in plants, and are important in photosynthesis. For human life, Chl's are constituents of the diet, especially in the form of green vegetables and fruits (*3*). Chlorophylls or their derivatives have also been used as additives for food-coloration purposes (*4*). In the pharmaceutical sciences, Chl has been made available as an over-the-counter drug such as chlorophyllin (Chlin; a semisynthetic, water-soluble sodium–copper salt derivative of chlorophyll); such a drug type has been widely used for controlling body, fecal, and urinary odor among geriatric patients (*5*).

Chlin has been found to exhibit antioxidant activity by way of inhibition of lipid peroxidation in rat liver (*6*, *7*), and also by means of its ability to protect mitochondria from oxidative damage induced by various reactive oxygen species (ROS) (*8*). Chlin has also been shown to inhibit radiation-induced DNA and mitochondrial membrane damage (*9*), and it would also appear to be a potent protector of DNA with regard to oxidative damage (*10*). Recently, Chlin has been used in cancer-related studies (*11*).

Natural Chl's deriving from plant leaves might be degraded into different forms. For example, chlorophyll *a* and *b* may be dephytylated by chlorophyllase in vivo to form chlorophyllide (Chlide) *a* and *b*, respectively. Further, Chlide *a* and *b* may lose magnesium to form pheophorbide (Pho) *a* and *b*. These compounds might be termed chlorophyll-related compounds (CRCs). Earlier studies have focused on the digestion and absorption of Chl's (*12*, *13*). Chl's may be digested to

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pheophytin and then absorbed within the micelle fraction according to the caco-2 human-cell model (*12*). Within the myeloma cell, the uptake of Chl's was converted into Pho (*13*). For the rabbit, both Chlide and Pho are the final metabolites of Chl's (unpublished data by Yang).

A number of earlier studies have indicated that vegetable extracts consisting of a greater number of Chl derivatives reveal a more positive relationship to the ability to inhibit mutations in the Ames salmonella system (*14, 15*), and also a greater level of anitgenotoxicity in the *Drosophila* wing spot test (*16*). Furthermore, animal studies have also shown that the application of Pho was effective with regard to both antitumor and anti-inflammation activities for ICR mouse skin (*17*), as well as acting as photodynamic sensitizers against tumors for mice (*18, 19*).

The antimutagenic effect of CRCs has been well documented. Recently, limited data appeared on the antioxidative capacity of these compounds in a tissue culture model. In this experiment, CRCs (Chlide *a* and *b* and Pho *a* and *b*) were tested for their antioxidative capacities in the context of their ability to protect (human) lymphocyte DNA from hydrogen peroxide (H_2O_2) induced DNA damage. Measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) is the commonest method of assessing DNA damage, but there is no consensus on what the true levels are in human DNA (*20*). Here, we measured DNA oxidative damage, both by the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG; test for oxidized base radical) and by way of the comet assay (test for single-strand breaks). The comet assay can be applied directly to the cell and measured DNA strand breaks.

MATERIALS AND METHODS

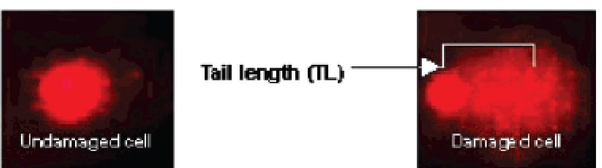
Chemicals. The chemicals used in these experiments were purchased from the following suppliers: agarose, Triton-100, dimethyl sulfoxide, and EDTA diodium salt dihydrate from J. T. Baker, USA; sodium chloride, hydrogen peroxide, potassium chloride, Trizma base, glucose, *N*-lauroylasarcosine, ethidium bromide (EtBr), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma, USA; low-melting-point agarose from BDH, UK. Frosted microscope slides were acquired from Fisher Scientific, USA.

Chlorophyll Related Compound Extracts. Chlorophyll-related compounds (CRCs) were obtained from spinach purchased in Taipei, Taiwan. It was prepared as previously described (*21*). Chlorophyll *a* and *b* were purified from spinach and dephytylated by the catalysis of chlorophyllase isolated from plant (*Ficus macrocarpa*) leaf, to form Chlide *a* and *b*, respectively. Chlide *a* and Chlide *b* were further magnesium (Mg) dechelated to form Pho *a* and Pho *b*, respectively.

Isolated Human Peripheral Blood Lymphocytes. Blood samples (10 mL) were obtained from healthy donors, and lymphocytes were isolated using a separation solution kit (Ficoll-Paque Plus lymphocyte isolation sterile solution; Pharmacia Biotech, Sweden.) For the experimental procedure, cells were harvested within 1 day of blood samples having been taken, and cultured with AIM V medium (serum-free lymphocyte medium; Gibco Invitrogen, USA) in a humidified atmosphere of 5% CO_2 in air at 37 °C for 24 h.

Treatment Procedures and Cell-Viability Testing. After culture, lymphocytes were exposed to one of four different CRCs (Chlide *a* and *b*, Pho *a* and *b*, with Chlin as a positive control), each of which was used at three concentrations (5, 20, or 50 μM) for 30 min at 37 °C. DNA damage was induced by exposing lymphocytes to H_2O_2 (10 or 50 μM) for 5 min on ice. Treatment on ice minimizes the possibility of cellular DNA repair after H_2O_2 injury. Cells were centrifuged (100*g* for 10 min), washed, and resuspended in the same medium for the comet assay and the assessment of 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in DNA. All experiments were carried out in triplicate. Cell viability was tested using the MTS assay (*22*), both prior to and after either CRC or H_2O_2 treatment.

DNA Single-Strand-Break Damage Estimation Using the Comet Assay. DNA single-strand break (ssb) damage was determined using the comet assay (*23*). Cultured lymphocytes (~10^5 cells/mL) were embedded in 75 μL of 1% low-melting-point agarose on a microscope slide (precoated with agarose) at 37 °C. The gel was allowed to set at 4 °C, and cells were lysed for a period of at least 2 h in lysis buffer at 4 °C. Cells were then alkaline-unwound, following which electrophoresis was carried out using the electrophoresis buffer at 4 °C for 15 min at 25 V with the current adjusted to 300 mA. All steps were conducted under dim light to prevent the occurrence of additional DNA damage. Following electrophoresis, slides were neutralized with neutralization buffer and stained with ethidium bromide. Under a fluorescence microscope (Nikon COOLPIX5000), the cometlike images resulting from the extension of DNA were scored as a reflection of the single-strand breaks. Duplicate slides were prepared for each experimental point sample, and 50 cometlike images selected at random per slide were evaluated to determine average DNA damage values. A computerized image analysis system (VisCOMET 1.3, Impuls, Germany) was employed to determine various comet parameters such as tail moment. The tail moment (integrated value of DNA density multiplied by the DNA migration distance) was used as the primary measure of DNA damage (*24*) (**Figure 1**).



TL = tail length

%DNA = DNA of tail given as a percentage

Figure 1. Under a fluorescence microscope, the cometlike images resulting from the extension of DNA were scored as a reflection of the single-strand breaks. The tail moment was defined as follows: TM = TL × % DNA.

Measurement of Oxidized Nucleoside Level by 8-Hydroxy-2′-deoxyguanosine (8-OHdG). Lymphocyte genomic DNA was purified using a genomic DNA purification kit (Bertec Enterprise, Taiwan), while 8-OHdG in lymphocyte DNA was quantified using an ELISA kit (Japan Institute for the Control of Aging, Japan). Briefly, samples or standards of 8-OHdG (0.5, 2, 8, 20, 80, and 200 ηg/mL) were placed in microtiter plates (8 × 12 wells; split type), which had been precoated with 8-OHdG. A monoclonal primary antibody recognizing 8-OHdG was added, and the plates were incubated for 1 h at 37 °C. The plates were then rinsed with PBS and subsequently incubated with an enzyme-labeled secondary antibody for a period of 1 h at 37 °C. Following this, plates were rinsed with PBS, and the chromatic substrate 3,3′,5,5′-tetramethylbenzidine was added, and incubation was carried out for 15 min at 37 °C in the dark. The reaction was terminated by the addition of phosphoric acid, and a further 3 min later, plates were read on a Microplate Reader that measured the absorbance at 450 nm. A standard curve was established by plotting the measured absorbance versus the logarithm of the concentration of 8-OHdG standards. Results were expressed as nanograms per microgram of DNA.

Measurement of DPPH Radical Scavenging. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method previously reported (*25*) was modified as follows: CRCs were dissolved in ethanol prior to reaction with a methanolic solution of DPPH (10 mM). The decrease in absorbance at 515 nm was determined with continuous data capture at 30-min intervals using a spectrophotometer (Hitachi U-3000). The DPPH scavenging capacity of CRCs was expressed as a proportional (percentage) inhibition (% inhibition), and was determined by the following expression:

$$\%\ \text{inhibition} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the sample at time = 0 and A_{sample} is the absorbance of the sample at time = 30 min.

Statistical Analyses. Data are reported as the mean ± standard deviation (SD) of triplicate determinations. Statistical analyses were

Table 1. Effect of CRCs on Human Lymphocyte Viability and DNA Damage[a]

	viability[b] (%)	DNA damage: comet assay (TM)[c]	DNA damage: 8-OHdG (ηg/μg of DNA)
chlorophyllin			
5 μM	98.5 ± 2.3	110 ± 12	0.53 ± 0.08
20 μM	96.4 ± 2.2	138 ± 38	0.53 ± 0.03
50 μM	95.4 ± 6.1	113 ± 22	0.63 ± 0.05
chlorophyllide *a*			
5 μM	97.5 ± 6.4	113 ± 22	0.53 ± 0.08
20 μM	98.4 ± 5.3	136 ± 30	0.68 ± 0.08
50 μM	96.2 ± 4.3	120 ± 35	0.60 ± 0.04
chlorophyllide *b*			
5 μM	98.8 ± 2.2	120 ± 31	0.47 ± 0.10
20 μM	96.8 ± 3.5	126 ± 55	0.79 ± 0.09
50 μM	99.2 ± 4.4	104 ± 36	0.61 ± 0.08
pheophorbide *a*			
5 μM	95.8 ± 6.5	86 ± 32	0.66 ± 0.05
20 μM	96.9 ± 3.4	100 ± 25	0.55 ± 0.04
50 μM	99.4 ± 2.5	99 ± 26	0.46 ± 0.07
pheophorbide *b*			
5 μM	98.4 ± 1.6	95 ± 20	0.63 ± 0.08
20 μM	99.7 ± 3.3	91 ± 17	0.62 ± 0.08
50 μM	99.8 ± 2.6	100 ± 31	0.43 ± 0.10
control	100	100 ± 21	0.55 ± 0.06

[a] Mean ± SD. [b] Viability (measured by the MTS assay) was determined both prior to (100%) and following CRC pretreatment. [c] Mean tail moment (TM) was calculated by means of the comet assay.

Table 2. Effect of H_2O_2 on Human Lymphocyte Viability and DNA Damage[a]

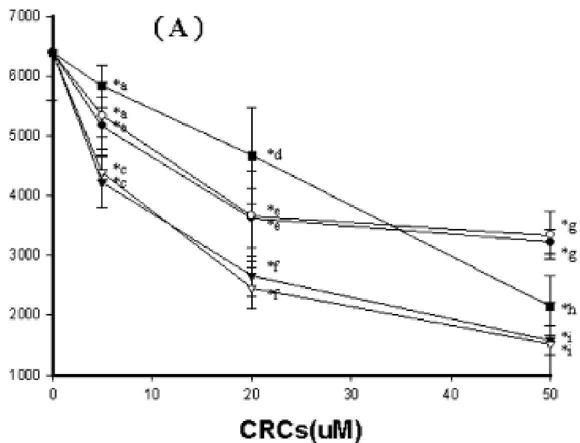
	viability[b] (%)	DNA damage: comet assay (TM)[c]	DNA damage: 8-OHdG (ηg/μg of DNA)
H_2O_2			
10 μM	99.4 ± 2.4	6386 ± 803[d]	2.30 ± 0.13[d]
50 μM	98.2 ± 7.1	14537 ± 1692[e]	3.98 ± 0.33[e]
control	100	100 ± 21[f]	0.55 ± 0.06[f]

[a] Mean ± SD. [b] Viability (measured by the MTS assay) was determined both prior to (100%) and following CRC pretreatment; no significant difference with regard to cell viability was apparent. [c] Mean tail moment (TM) was calculated for the comet assay. [d–f] Values featuring different letters differ significantly with regard to H_2O_2 levels (ANOVA, $p < 0.05$).

performed using a Student's *t*-test to compare differences between control and CRC-pretreated groups. One-way ANOVA was used to test for differences among the CRC groups. Post hoc comparison of means was performed by Duncan's multiple comparison, and $p < 0.05$ was considered to represent a statistically significant difference between test populations.

RESULTS

The initial viability of, and the level of DNA damage incurred by human lymphocytes from this experimental procedure, is revealed in **Table 1**. When human lymphocytes were pretreated with each of these CRCs, the subsequent cell viability did not change significantly, nor did the level of cell death or oxidative DNA damage compared with the solvent control. Following free-radical attack, the viability of and DNA damage sustained by human lymphocytes were determined, and the data are summarized in **Table 2**. The viability of human lymphocytes at the experimental procedure also did not differ significantly from control. When cells were challenged with free radicals, however, a significant increase in DNA damage was noticed compared to the control. The effect of lymphocyte exposure to 10 μM H_2O_2 was 60-fold (from 100 to 6386) greater with regard



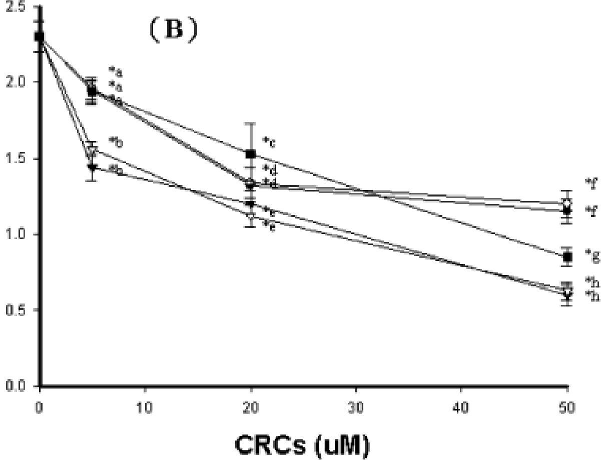
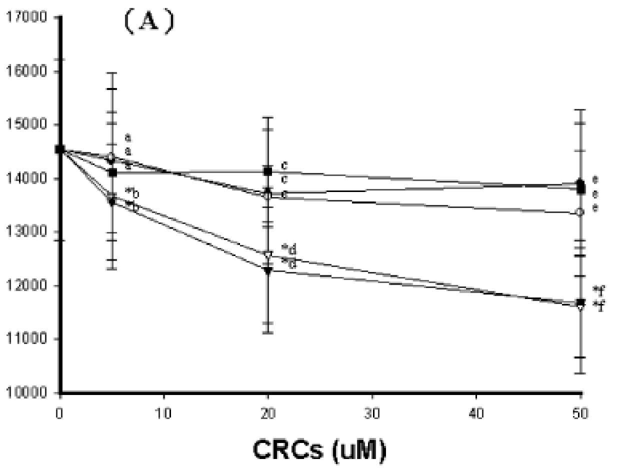


Figure 2. Effect of CRC pretreatment on 10 μM hydrogen peroxide induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the comet assay (**A**) and 8-OHdG levels in lymphocyte DNA (**B**). Results are the mean ± SD. (●, chlorophyllide *a*; ○, chlorophyllide *b*; ▼, pheophorbide *a*; ▽, pheophorbide *b*; ■, chlorophyllin.) Values with different letters differ significantly with regard to oxidative damage when comparing between different CRCs (ANOVA, $p < 0.05$); *$p < 0.05$ refers to differences in oxidative damage as compared with the control (without CRCs).

to DNA ssb damage than was the case for the control, and a significantly enhanced level of 8-OhdG adducts (from 0.55 to 2) was also observed. When lymphocytes were exposed to 50 μM H_2O_2, the level of oxidative damage increased significantly as revealed by the level of ssb's (from 100 to 14 537) and oxidized nucleoside formation (8-OHdG from 0.55 to 3.98), when compared to the control.

The effect of CRC pretreatment on 10 μM H_2O_2 induced DNA damage in human lymphocytes is presented in **Figure 2**. For each concentration of CRC-pretreated cells, a significantly reduced level of DNA ssb's and 8-OHdG formation following H_2O_2 exposure was observed ($p < 0.05$). It may also be seen from this figure that the protective effect on lymphocytes of pretreatment of such cells with each of the CRCs at the lower dose for each (5−20 μM) occurred in a dose-dependent manner. Those lymphocytes pretreated with Pho *a* or *b* (5−50 μM) experienced a greater level of protection against H_2O_2 exposure than did lymphocytes exposed to the other test compounds, and in a dose-dependent manner. The maximum protective effect for lymphocyte pretreatment was seen for pretreatment with 50 μM Pho *a* and *b* (respectively 75.28% and 76.17% inhibition



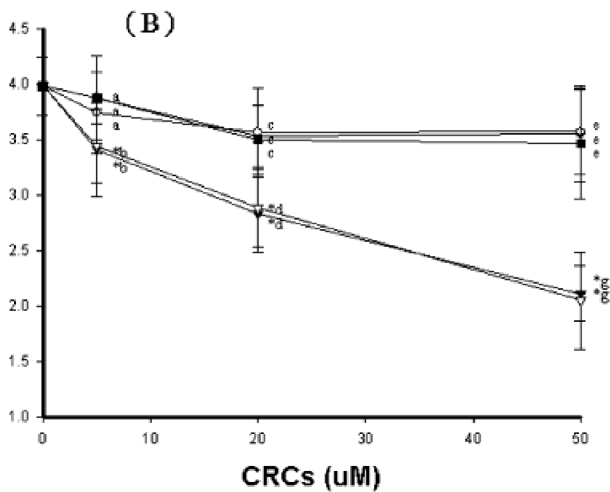


Figure 3. Effect of CRC pretreatment on 50 μM hydrogen peroxide induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the comet assay (**A**) and 8-OHdG levels in lymphocyte DNA (**B**). Results are the mean ± SD. (●, chlorophyllide *a*; ○, chlorophyllide *b*; ▼, pheophorbide *a*; ▽, pheophorbide *b*; ■, chlorophyllin.) Values with different letters differ significantly with regard to oxidative damage when comparing among different CRCs (ANOVA, $p < 0.05$); *$p < 0.05$ refers to differences in oxidative damage as compared with the control (without CRCs).

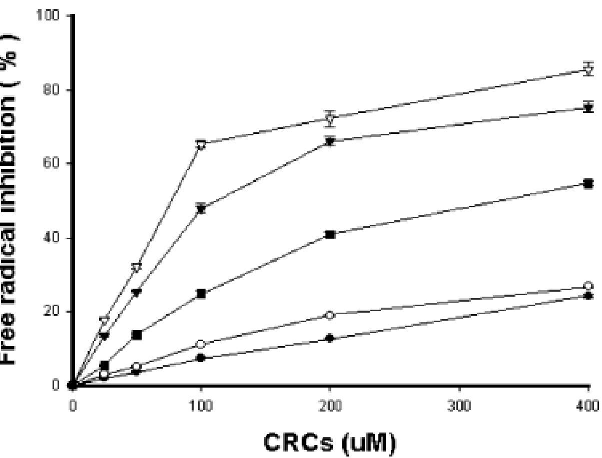


Figure 4. DPPH scavenging capacity of CRCs. The absorbance inhibition for DPPH was monitored at 515 nm. Results represent the mean ± SD. (●, chlorophyllide *a*; ○, chlorophyllide *b*; ▼, pheophorbide *a*; ▽, pheophorbide *b*; ■, chlorophyllin.)

compared with the solvent control; **Figure 2A**). A similar result was noted when the level of Pho *a* or *b* protection to H_2O_2 exposure was assessed in the context of the production of 8-OHdG adducts (**Figure 2B**).

The protective effect of CRC pretreatment on 50 μM H_2O_2 induced DNA damage for human lymphocytes is presented in **Figure 3**. At a 50 μM H_2O_2 challenge, both Chlin and Chlide failed to demonstrate any degree of protection against DNA damage and the level of damage for both cases was similar to that of the positive control. With regard to Pho *a* and *b*, both expressed antioxidative protective effects at all of the concentrations tested: pretreatment with such agents elicited a decrease in DNA ssb's (19.63% and 20.18% inhibition compared with the solvent control) and 8-OHdG adduct formation following H_2O_2 exposure (**Figure 3**). The lymphocytes pretreated with either Pho *a* or *b* exhibited protection against not only low doses of H_2O_2 exposure, but also high doses of H_2O_2-induced DNA damage.

The free-radical scavenging capacity of CRCs is shown in **Figure 4**. Four CRCs were tested; they exhibited diverse antioxidative capacities regarding their ability to scavenge the DPPH free radical. The scavenging capacities of Pho did appear to be more pronounced than that of Chlide's. The IC_{50} value for DPPH inhibition of Pho *b*, Pho *a*, and Chlin were, respectively, 75, 120, and 360 μM, while the corresponding figures for Chlide *a* and *b* were somewhat greater than 800 μM.

From DNA damage and free-radical scavenging tests, CRCs had antioxidative effects and the composition of magnesium ion in CRCs might affect their antioxidative ability under either ex vivo or in vitro stress conditions.

DISCUSSION

Exposure to an adverse aerobic environment can elicit the generation of reactive oxygen species (ROS) resulting in oxidative stress (*26*). Excess ROS generation can significantly alter the structural and functional relationships of biomolecules and has been implicated as a cause of oxidative DNA damage (*27*). Chlin has been reported to exhibit a greater level of antimutagenic activity than the well-known suite of antioxidant vitamins including retinal, β-carotene, vitamin E, and vitamin C (*28*). Furthermore, Chlin's antioxidant ability is more substantial than that of vitamin C, glutathione, and mannitol (*8*). Natural chlorophylls, in contrast, have been little studied in this regard (*16*). Our results showed that Chl's from water extract of spinach significantly reduced single-strand-break formation and 8-OHdG adduct level; furthermore, Pho *a* and *b* experienced a greater level of protection than Chlin. The results might explain partially why vegetables containing chlorophyll derivatives would protect against and reduce the risk of ROS-related diseases.

The basic structure of CRCs contains a porphyrin-related chlorine ring skeleton, which possesses more than 10 conjugative double bonds, and appears to be a responsible chemical structure for antioxidant activity (*29*). We examined the free-radical scavenging capacity of CRCs, which quenched DPPH (10 mM) to process a stable free radical. Endo et al. (*30*) indicated that CRCs provide the hydrogen donor to reduce free radicals such as DPPH, and identified a π-cation radical of porphyrin compounds when CRCs react with free radical using electron spin resonance spectroscopy. Hydrogen peroxide is believed to cause DNA oxidative damage by generation of hydroxyl radical via the Fenton reaction. For the present study, we remain

uncertain regarding the precise quantity of ferric ions in human lymphocyte to induce hydroxyl radical generation, but we compare the composition of the water-soluble CRCs investigated in the present study: Chlin contains a copper ion in its porphyrin structure and Chlide contains magnesium ion while Pho differs markedly in that such molecules do not contain any metal ions in the porphyrin ring. It remains possible that Pho acts by way of an ion-chelation effect to reduce metal-induced hydroxyl radical generation, thus protecting DNA from oxidative damage (*31*). We speculate that both Pho *a* and *b* exert their antioxidative capacity not only by the porphyrin stabilization of ROS, but also by their inherent ion-chelation capacity.

In conclusion, the water-extract-Chl derivatives reduce the DNA damage induced by hydrogen peroxide in isolated human lymphocytes. Pho compounds were more effective than Chlide. These compounds might act as free-radical scavengers and as chelating agents to protect human lymphocyte DNA from oxidative damage.

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LITERATURE CITED

(1) Phillips, R. L. Role of life-style and dietary habits in risk of cancer among Seventh-Day Adventists. *Cancer Res.* **1975**, *35*, 3513−3522.
(2) Block, G.; Patterson, B.; Subar, A. Fruits, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* **1992**, *18*, 1−29.
(3) Schwartz, S. J.; Lorenzo, T. V. Chlorophylls in foods. *Crit. Rev. Food Sci. Nutr.* **1990**, *29*, 1−17.
(4) Timberlake, C. F.; Henry, B. S. Plant pigments as natural food colours. *Endeavour* **1986**, *10*, 31−36.
(5) Young, R. W.; Beregi, J. S. Use of chlorophyllin in care of geriatric patients. *J. Am. Geriat. Soc.* **1980**, *24*, 46−47.
(6) Sato, M.; Imai, K.; Murata, T. Effect of sodium copper chlorophyllin on lipid peroxidation: The antioxidative activities of the commercial preparations of sodium copper chlorophyllin. *Yakugaku Zasshi* **1980**, *100*, 580−584.
(7) Sato, M.; Iguchi, N.; Murata, T. Effect of sodium copper chlorophyllin on lipid peroxidation: I. Effect on lipid peroxidation in rat homogenates in the presence of both Fe^{2+} and L-ascorbic acid. *Yakugaku Zasshi* **1977**, *97*, 268−273.
(8) Kamat, J. P.; Boloor, K. K.; Devasagayam, T. P. Chlorophyllin as an effective antioxidant against membrane damage in vitro and ex vivo. *Biochim. Biophys. Acta* **2000**, *1487*, 113−127.
(9) Boloor, K. K.; Kamat, J. P.; Devasagayam, T. P. Chlorophyllin as a protector of mitochondrial membranes against gamma-radiation and photosensitization. *Toxicology* **2000**, *155*, 63−71.
(10) Kumar, S. S.; Chaubey, R. C.; Devasagayam, T. P. A.; Priyadarsini, K. I.; Chauhan, P. S. Inhibition of radiation-induced DNA damage in plasmid pBR322 by chlorophyllin and possible mechanism(s) of action. *Mutat. Res.* **1999**, *425*, 71−79.
(11) Egner, P. A.; Wang, J. B.; Zhu, Y. R.; Zhang, B. C.; Wu, Y.; Zhang, Q. N.; Qian, G. S.; Kuang, S. Y.; Gange, S. J.; Jacobson, L. P.; Helzlsouer, K. J.; Bailey, G. S.; Groopman, J. D.; Kensler, T. W. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. *Proc. Natl. Acad. Soc. U.S.A.* **2001**, *98*, 14601−14606.
(12) Ferruzzi, M. G.; Failla, M. L.; Schwartz, S. J. Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and caco-2 human cell model. *J. Agric. Food Chem.* **2001**, *49*, 2082−2089.
(13) Chernomorsky, S.; Segelman, A.; Poretz, R. D. Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth. *Teratog. Carcinog. Mutagen.* **1999**, *19*, 313−322.
(14) Lai, C. N.; Butler, M. A.; Matney, T. S. Antimutagenic activities of common vegetables and their chlorophyll content. *Mutat. Res.* **1980**, *77*, 245−250.
(15) Kimm, S. W.; Tchai, B. S.; Kang, S. Antimutagenic activity of chlorophyll to direct- and indirect-acting mutagens and its contents in the vegetable. *Korean J. Biochem.* **1982**, *14*, 1−7.
(16) Negishi, T.; Rai, H.; Hayatsu, H. Antigenotoxic activity of natural chlorophylls. *Mutat. Res.* **1997**, *376*, 97−100.
(17) Nakamura, Y.; Murakami, A.; Koshimizu, K.; Ohigashi, H. Inhibitory effect of pheophorbide a, a chlorophyll-related compound on skin tumor promotion in ICR mouse. *Cancer Lett.* **1996**, *108*, 247−255.
(18) Pandey, R. K.; Bellnier, D. A.; Smith, K. M.; Dougherty, T. J. Chlorin and porphyrin derivatives as potential photosensitizers in photodynamic therapy. *Photochem. Photobiol.* **1991**, *53*, 65−72.
(19) Pandey, R. K.; Shiau, F. Y.; Sumlin, A. B.; Dougherty, T. J.; Smith, K. M. Structure/activity relationships among photosensitizers related pheophorbides and bacteriopheophorbides. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 491−496.
(20) Halliwell, B. Why and how should we measure oxidative DNA damage in nutritional studies? How far have we come? *Am. J. Clin. Nutr.* **2000**, *72*, 1082−1087.
(21) Yang, C. M.; Chang, K. W.; Yin, M. H.; Huang, H. M. Methods for the determination of the chlorophylls and their derivatives. *Taiwania* **1998**, *43*, 116−122.
(22) Cory, A. H.; Owen, T. C.; Barltrop, J. A.; Cory, J. G. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* **1991**, *3*, 207−212.
(23) Singh, N. P.; McCoy, M. T.; Tice, R. R.; Schneider, E. L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **1988**, *175*, 184−191.
(24) Fairbairn, D. W.; Olive, P. L.; O'Neill, K. L. The comet assay: a comprehensive review. *Mutat. Res.* **1995**, *229*, 37−59.
(25) Sandoval, M.; Okuhama, N. N.; Angeles, F. M.; Melchor, V. V.; Condezo, L. A.; Lao, J.; Miller, M. J. S. Antioxidant activity of the cruciferous vegetable Maca. *Food Chem.* **2002**, *79*, 207−213.
(26) Sies, H. Biochemistry of oxidative stress. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1058−1071.
(27) Halliwell, B.; Gutteridge, J. M. C. *Free radicals in biology and medicine*, 3rd ed.; Oxford University Press: Oxford, UK, 1999.
(28) Ong, T. M.; Whong, J.; Stewart, J.; Brockman, H. E. Comparative antimutagenicity of 5 compounds against 5 mutagenic complex mixture in Salmonella typhimurim stain TA 98. *Mutat. Res.* **1989**, *222*, 19−25.
(29) Matsushita, S.; Iwami, N. Antioxidative abilities of some porphyrins on the oxidation of sodium linoleate. *Arch. Biochem. Biophys.* **1965**, *112*, 476−477.
(30) Endo, Y.; Usuki, R.; Kaneda, T. Antioxidant effects of chlorophyll and pheophytin on the autoxidation of oil in the dark. II. The mechanism of antioxidative action of chlorophyll. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1387−1390.
(31) Longo, F. R.; Brown, E. M.; Quimby, D. J. Kinetic studies on metal chelation by porphyrins. *Ann. NY Acad. Sci.* **1973**, *206*, 420−442.

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• *BASIC RESEARCH* •

Consumption of purple sweet potato leaves modulates human immune response: T-lymphocyte functions, lytic activity of natural killer cell and antibody production

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Abstract

AIM: To study the immunological effects of physiological doses of purple sweet potato leaves (PSPL).

METHODS: The randomized crossover study (two periods, each lasting for 2 wk) involved 16 healthy non-smoking adults of normal weight. The 6-wk study consisted of a run-in (wk 1) PSPL diet (daily consumption of 200 g PSPL) or a control diet (low polyphenols, with the amount of carotenoids adjusted to the same level as that of PSPL) (wk 2-3), washout diet (wk 4), and switched diet (wk 5-6). Fasting blood was collected weekly in the morning. T-lymphocyte function was assessed via the proliferation and secretion of immunoreactive cytokines. Salivary IgA secretion and the specific cytotoxic activities of cytotoxic T lymphocytes and natural killer (NK) cells were determined.

RESULTS: The plasma β-carotene level increased with time in both groups, while the plasma polyphenol level decreased in the control group, and no significant difference was detected between the two groups. Although plasma polyphenol levels did not significantly increase in the PSPL group at the end of the study, they were significantly elevated in urine. PSPL consumption produced a significant increase in proliferation responsiveness of peripheral blood mononuclear cells (PBMC) and their secretion of immunoreactive IL-2 and IL-4. As well, lytic activity in NK cells was elevated in a time-dependent fashion. Salivary IgA secretion significantly decreased in control group after 2 wk, and returned to baseline following dietary switch to PSPL.

CONCLUSION: Consumption of PSPL modulates various immune functions including increased proliferation responsiveness of PBMC, secretion of cytokines IL-2 and IL-4, and the lytic activity of NK cells. The responsible determinants of PSPL remain to be elucidated, as does the biological significance of the present observations.

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Key words: Purple sweet potato leaves; Polyphenol; Immune function

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INTRODUCTION

The high consumption of vegetables and fruits has been linked epidemiologically to a decreased risk of cancer and cardiovascular disease[1]. Their beneficial effects have been attributed partly to the presence of numerous polyphenolic compounds, which display antioxidant and free radical scavenging properties[2]. Polyphenols are the major phytochemicals in fruits and vegetables. A variety of *in vitro* studies have shown that polyphenols such as flavonoids are antioxidants[3], immunomodulators[4], and exhibit antigenotoxic effects[5].

Purple sweet potato leaves (PSPL), which are easily grown in tropical areas such as Taiwan, have the highest polyphenolic content, in particular flavonoids, of all the commonly grown vegetables, and exhibit free radical scavenging ability[6]. Supplementation of diets with PSPL would seemingly be prudent. However, this recommendation is premature, since little is known from human experimental studies about the physiologic effects of dietary PSPL on the immune system.

As with many vegetables and fruits, PSPL are rich in carotenoids. The immunomodulatory activity of carotenoids in animals and human beings is well known[3-5]. Presently, we sought to evaluate whether physiological doses of polyphenol via consumption of PSPL affected the immune status of healthy subjects. In order to calibrate the

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immunomodulatory effect of carotenoids, the amount of carotenoids consumed by the test and control groups was similarly adjusted. The results are consistent with a beneficial immune effect of PSPL.

MATERIALS AND METHODS

Subjects

Sixteen non-smoking healthy adults (seven men and nine women, aged 20-22 years), with normal weight (body mass index 20.9-21.6 kg/m^2) were recruited for the study. All subjects were in good medical health. Their screening histories did not reveal indications of any gastrointestinal disorder. Vitamin supplements or medications were not taken 1 mo preceding the study and during the study period. The study was approved by Medical Ethical Committee of the Taipei Medical University and all participants gave their written consent.

Sample collection and processing

Study design The leaves used were obtained from purple sweet potatoes planted and tended at the Taoyuan District Agriculture Improvement Station, Taipei Branch, Taiwan.

The randomized crossover study was conducted during September to November 2003. Subjects were randomly divided into control group and experimental group, all of them ran in a low polyphenol diet for 1 wk (wk 1). After that, control group consumed a control diet (low polyphenols, with the amount of carotenoids adjusted to the same level as that of PSPL) for 2 wk, experimental group consumed a PSPL diet (daily consumption of 200 g PSPL) for 2 wk (wk 2-3). The washout diet (a low polyphenol diet) was followed for 1 wk (wk 4), and then, the control and experimental groups were given switched diet for 2 wk (wk 5-6). Results were presented in a total study period of 6 wk.

Subjects were provided PSPL and control diet for lunch and dinner by the Department of Dietetics of Taipei Medical University Hospital. The meals were eaten in the hospital cafeteria. Each subject returned any uneaten food to allow food intake to be recorded. A list of the food products that the subjects were not allowed to eat was provided. Each subject kept a record of food consumption, which was checked to ensure compliance with the diet.

Collection and preparation of blood samples Blood from fasting subjects was collected once weekly in the morning between 07.00 a.m. and 09.00 a.m. Blood was drawn from an antecubital vein into the tubes containing 1.6 g/L of EDTA or Li-heparin, which were immediately placed on ice in the dark. Plasma collected by centrifugation at 1 500 r/min for 10 min at 4 ℃ was stored at -80 ℃ until analysis.

Collection and preparation of urine samples Before and at the end of the intervention period all volunteers were asked to provide a 24-h urine sample. During the collection period urine was stored at 4 ℃ and kept in dark bottles. Exact sampling time and urine volume were determined, and each sample was stored at -20 ℃ until analysis.

Analytical methods

Total polyphenols in urine and plasma Total polyphenols in urine and plasma were measured using the Folin-Ciocalteau method[10]. The absorption at 750 nm was measured spectrophotometrically. The total polyphenol content was expressed as gallic acid equivalents (GAE).

Plasma β-carotene Carotenoids were extracted from plasma with absolute ethanol (containing 1% pyrogallol) and hexane, separated by C18 reverse phase high-pressure liquid chromatography using spherical 3 micron packing. β-carotene was identified and quantified using an external standard[11].

Preparation of peripheral blood mononuclear cells (PBMC) PBMC were isolated by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Buckinghamshire, UK) and resuspended in complete RPMI-1640 culture medium (Invitrogen, Carlsbad, CA, USA), containing 50 mL/L heat-inactivated fetal bovine serum (FBS, Invitrogen), 2 mmol/L L-glutamine, 100 000 U/L penicillin, and 100 mg/L streptomycin.

Lymphocyte proliferation PBMC (1×10^9 cells/L) in medium containing 5% of FBS were stimulated by T cell mitogen concanavalin A (Con A; 5 mg/L; Sigma Chemical Co., St. Louis, MO, USA) for 120 h at 37 ℃. Proliferation was measured using the BrdU proliferation enzyme immunoassay kit (Oncogene, Merck, Darmstadt, Germany).

Quantification of cytokine secretion PBMC were stimulated with Con A as described above for 48 h to measure IL-2 and IL-4 secretion. Cell-free supernatants were collected and stored at -80 ℃ until analysis. IL-2 and IL-4 were measured by ELISA kit (Biosource, Camarillo, CA, USA).

Cytotoxicity activity assay of NK cells The specific cytotoxic activities of natural killer (NK) cells were tested using a Promega CytoTox 96 kit (Promega, Madison, WI, USA). K562 cells were susceptible target cells to the cytotoxic effects of NK cells. K562 cells and PBMC including NK cells, resuspended in phenol red free RPMI-1640 culture medium containing 5% FBS were coincubated in 96-well round bottomed plates for 4 h at 37 ℃. The following PBMC/ K562 cell ratios 30:1, 20:1, 15:1, 10:1, and 7.5:1 were used to find a good effect between NK cells and K562 cells. During the incubation, the NK cells destroyed the target cells and lactate dehydrogenase (LDH) was released. Spontaneous release of PBMC or target cells was controlled by separate incubation of the respective population and detergent was added to lyse all target cells into a complete cytotoxicity. At the end of incubation, the cells were lysed and centrifuged. Fifty microliters of aliquot of each well was transferred into another 96 well flat-bottomed plate and 50 μL of fresh LDH substrate solution was added to each well. The plates were incubated at room temperature for 30 min, and the reaction was stopped by the addition of 1 mol/L acetic acid. The resulting light absorbance was measured in microplate reader at 490 nm. The percentage of cells exhibiting cytotoxic activity was calculated.

Salivary IgA secretion A small absorbent pad was used to collect saliva before brushing of the teeth in the morning. All samples were centrifuged at 13 000 *g* in a microliter tube. The supernatant was stored at -20 ℃ until analysis. Salivary immunoglobulin A (IgA)[12] was measured using an immunoassay kit (Salimetrics, State College, PA, USA).

Statistical analysis

All values were expressed as mean±SD. The data were analyzed using SPSS version. Statistical significance was determined using pairs *t* test. $P<0.05$ was considered statistically significant.

RESULTS

All participants tolerated the intervention well and completed the study. Physical characteristics of the subjects are summarized in Table 1. Body mass index and the percentage of body fat did not vary significantly during the study. PSPL-related daily dietary intake was 902 mg GAE of total polyphenol and 23.42 mg of β-carotene. Control subjects ingested the same amount of β-carotene from 40 to 45 g carrots (268.76 μg/g of β-carotene) each day.

The concentrations of plasma β-carotene and polyphenol measured throughout the study are summarized in Table 2. Plasma β-carotene level increased with time in the PSPL and control groups. However, plasma polyphenol levels decreased in the control group, although the decrease was not significantly different from the PSPL group. In the latter group, the urine levels of polyphenol were significantly elevated (8.20±0.95 to 10.27±1.39 mg GAE/dL).

Table 1 Physical characteristics of subjects (mean±SD)

Subjects	Before		After	
	Male	Female	Male	Female
n	7	9	7	9
Age (yr)	21±1.5	20±1.2	21±1.5	20±1.2
Height (cm)	175.4±5.7	161.9±7.2	175.4±5.7	161.9±7.2
Body weight (kg)	64.8±9.6	53.8±6.4	65.9±9.2	54.7±6.6
Body mass index (kg/m^2)	21.0±2.6	20.6±2.3	21.4±2.4	20.9±2.3
Body fat (%)	16.4±4.5	26.8±4.9	17.4±4.5	28.7±4.6

Table 2 Change of plasma and urine β-carotene and polyphenol levels in subjects (mean±SD)

	Baseline	Washout	1 wk	2 wk
β-carotene in plasma (μmol/L)				
Control	0.088±0.06	0.076±0.02	0.104±0.05	0.125±0.05[a]
PSPL	0.091±0.04	0.107±0.04	0.125±0.05[c]	0.139±0.04[c]
Polyphenol in plasma (mg GAE/dL)				
Control	61.61±1.39	61.04±1.94	58.86±1.38[c]	59.02±1.35[c]
PSPL	59.21±3.34	58.87±1.63	59.44±2.00	60.01±1.41
Polyphenol in urine (mg GAE/dL)				
Control	8.38±1.50	8.28±1.19	6.76±131[c]	6.95±1.36[c]
PSPL	8.20±0.95	8.25±1.58	10.30±0.89[c,e]	10.27±1.39[c,e]

[a]$P<0.05$ for 2 wk *vs* baseline, washout, 1 wk. [c]$P<0.05$ for baseline *vs* 1 and 2 wk. [e]$P<0.05$ for control *vs* PSPL.

Proliferation responsiveness of Con A-activated PBMC increased significantly after consumption of PSPL (Figure 1). Secretion of T-helper lymphocyte cytokine IL-2 increased after consumption of PSPL for 1 and 2 wk (Figure 2A). The ability of PBMC to secrete immunoreactive IL-4 also significantly increased during the same period (Figure 2B). Lytic activity of NK cells was also affected by PSPL consumption (Figure 3). The results showed that a PBMC/target cell ratio of 30:1 had the highest percentage of cytotoxicity, and the other ratios had the same trend but no

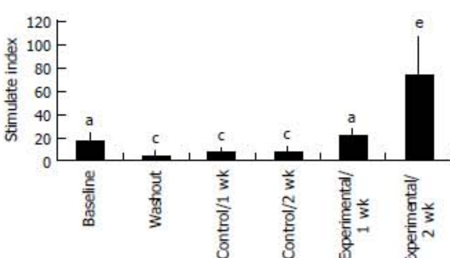

Figure 1 Proliferation responsiveness of PBMCs. [a]$P<0.05$ for baseline *vs* washout, control 1 and 2 wk. [c]$P<0.05$ for experimental 2 wk *vs* washout, control 1 and 2 wk. [e]$P<0.05$ for experimental 1 wk *vs* experimental 2 wk.

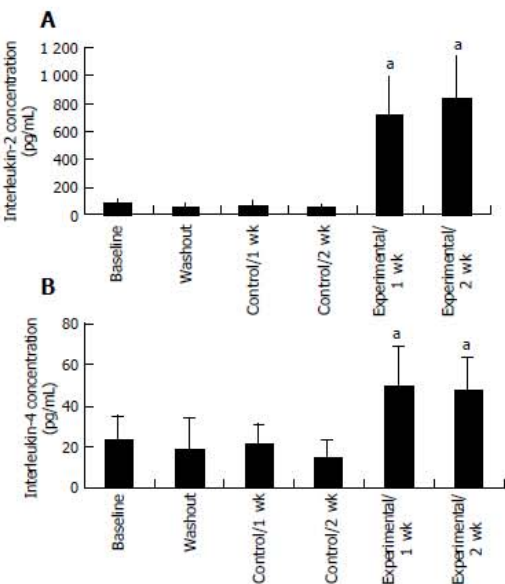

Figure 2 Concentration of interleukin-2 (**A**) and interleukin-4 (**B**) secreted by PBMCs. [a]$P<0.05$ for experimental 2 wk *vs* baseline, washout, control 1 and 2 wk.

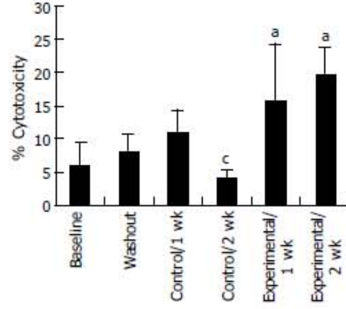

Figure 3 Lytic activity of NK cells. [a]$P<0.05$ for experimental 2 wk *vs* baseline, washout, control 1 and 2 wk. [c]$P<0.05$ for control 2 wk *vs* washout, control 1 wk, experimental 1 and 2 wk.

5780 ISSN 1007-9327 CN 14-1219/ R World J Gastroenterol October 7, 2005 Volume 11 Number 37

significance (data not shown). The percentage of lytic activity of NK cells in a PBMC/target cell ratio of 30:1 was elevated in the PSPL group after 1 wk, and then was further elevated. Salivary IgA secretion in the control group decreased significantly, but returned to the basal levels after 2 wk of PSPL consumption (Figure 4).

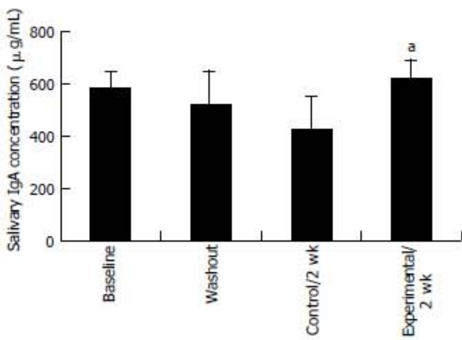

Figure 4 Concentration of salivary IgA detected by immunoassay kit. $^aP<0.05$ for experimental 1 wk *vs* experimental 2 wk.

DISCUSSION

The present study demonstrates that consumption of polyphenol-rich PSPL results in a marked total polyphenol increase in the urine. Most polyphenols peak in plasma 1-2 h after a single polyphenol intake, and are quickly eliminated from the circulation[13]. The prolonged ingestion of polyphenol-rich PSPL did not result in increased plasma polyphenol concentrations as expected. We also measured the total polyphenol in plasma 0, 2, 4, 8, 12, 24 h after consumption of PSPL in a previous study. The plasma polyphenol level raised 2 and 4 h after consumption of PSPL and further increased after 24 h, but there was no significance (data not shown). Although polyphenol was not different in plasma from fasting subjects throughout the whole study period, physiological functions were significantly modified.

β-Carotene-rich carrots were used to calibrate the effect of carotenoids from PSPL. As expected, β-carotene concentration in plasma was not significantly different. In another study, the dietary supplementation with β-carotene (8.2 mg) or lycopene (13.3 mg) for 12 wk does not enhance cell-mediated immunity in healthy free-living elderly humans beings[14]. A low-carotenoid diet reduces T-lymphocyte function, which cannot be restored by the daily supplementation with carrot juice containing 21.6 mg β-carotene, 15.7 mg α-carotene, and 0.5 mg lutein[9]. Presently, control subjects ingesting 23.42 mg of β-carotene each day do not display a significantly altered T-lymphocyte function.

The immune system provides markers for determining the biological health benefits of phytochemicals. Flavonoids suppress a variety of immune functions including lymphocyte proliferation, lytic activity of NK cells, and cytokine secretion *in vitro*[4]. The same study demonstrated that supplementing a low polyphenol diet with polyphenol-rich fruit juices results in significantly increased lymphocyte proliferative responsiveness, IL-2 secretion by activated lymphocytes, and the lytic activity of NK cells in healthy subjects[17]. These results are in agreement with the findings in our present study.

Many health benefits are associated with the consumption of tea, and are mainly attributed to the polyphenolic constituents. In animals, these constituents exert antioxidative activities and improve the antidotal capability of the liver. We found similar oxidative effects following the 2-wk regimen of PSPL consumption in humans beings (data not shown). Free radicals impair the integrity and functionality of membrane lipids and affect signal transduction and gene expression in immune cells[18]. One potential mechanism underlying the immunomodulatory effect of polyphenols is their ability to act as an antioxidant, resulting in a lower generation of free radicals.

IL-2 and IL-4 are both produced by activated T cells following *in vitro* stimulation by T-cell mitogens such as Con A. IL-2 is primarily produced by T-helper-1 cells, and IL-4 by T-helper-2 cells[19]. In our study, consumption of polyphenol-rich PSPL resulted in increased secretion of IL-2 and IL-4, which paralleled an enhanced proliferative response of these cells.

Pure flavonoids suppress a variety of immune mechanisms including lymphocyte proliferation, lytic activity of NK cells *in vitro*[4]. In animal models, quercetin also increases lytic activity of NK cells[20]. A study of healthy human subjects clearly showed that supplementing a low-polyphenol diet with polyphenol-rich fruit juices significantly increases lymphocyte responsiveness to mitogen activation and enhances lytic activity of NK cells[17]. The present findings are entirely consistent with the prior observations and support the suggestion that polyphenol possesses immunoactivity *in vivo* that differs from the activity observed *in vitro*. Possibly the polyphenol doses used *in vitro* are different from polyphenol in plasma concentrations and/or have the specific function of the metabolites of polyphenol.

IgA is the most important immunoglobulin in saliva, serving as a main immunological defense of mucosal surfaces[16]. Salivary IgA is a sensitive indicator of the humoral immune response to infection[17]. In our study, salivary IgA secretion significantly decreased when subjects consumed low polyphenol diet and returned to the basal level after PSPL consumption, indicating that PSPL consumption may protect mucosal surfaces.

In conclusion, dietary intervention in the form of PSPL consumption can modulate various immune functions. The active constituents in PSPL need to be determined in further investigations.

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REFERENCES

1 Block G, Patterson B, Subar A. Fruit, vegetables, and cancer

prevention: a review of the epidemiological evidence. *Nutr Cancer* 1992; **18**: 1-29
2 **Surh Y**. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mut Res* 1999; **428**: 305-327
3 **Rice-Evans CA**, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad Biol Med* 1996; **20**: 933-956
4 **Middleton E Jr**. Effect of plant flavonoids on immune and inflammatory cell function. *Adv Exp Med Biol* 1998; **439**: 175-182
5 **Duthie SJ**, Johnson W, Dobson VL. The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimdines) and growth in human cells. *Mut Res* 1997; **390**: 141-151
6 **Chu YH**, Chang CL, Hsu HF. Flavonoid content of several vegetables and their antioxidant activity. *J Sci Food Agr* 2000; **80**: 561-566
7 **Bendich A**. Carotenoids and the immune response. *J Nutr* 1989; **119**: 112-115
8 **Kramer TR**, Burri BJ. Modulated mitogenic proliferative responsiveness of lymphocytes in whole-blood cultures after a low-carotene diet and mixed-carotenoid supplementation in women. *Am J Clin Nutr* 1997; **65**: 871-875
9 **Watzl B**, Bub A, Brandstetter BR, Rechkemmer G. Modulation of human T-lymphocyte functions by the consumption of carotenoid-rich vegetables. *Br J Nutr* 1999; **82**: 383-389
10 **Pearson DA**, Tan CH, German JB, Davis PA, Gershwin ME. Apple juice inhibits human low density lipoprotein oxidation. *Life Sci* 1999; **64**: 1913-1920
11 **Gatautis VJ**, Pearson KH. Separation of plasma carotenoids and quantitation of beta-carotene using HPLC. *Clin Chim Acta* 1987; **166**: 195-206
12 **Koh D**, Yang Y, Khoo L, Nyunt SZ, Ng V, Goh CL. Salivary immunoglobulin A and lysozyme in patients with psoriasis. *Ann Acad Med* 2004; **33**: 307-310
13 **Scalbert A**, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr* 2000; **130**: 2073S-2085S
14 **Corridan BM**, O'Donoghue M, Hughes DA, Morrissey PA. Low-dose supplementation with lycopene or beta-carotene does not enhance cell-mediated immunity in healthy free-living elderly humans. *Eur J Clin Nutr* 2001; **55**: 627-635
15 **Mestecky J**. Saliva as a manifestation of the common mucosal immune system. *Ann N Y Acad Sci* 1993; **694**: 184-194
16 **Ludwig K**, Grabhorn E, Bitzan M, Bobrowski C, Kemper MJ, Sobottka I, Laufs R, Karch H, Muller-Wiefel DE. Saliva IgM and IgA are a sensitive indicator of the humoral immune response to Escherichia coli O157 lipopolysaccharide in children with enteropathic hemolytic uremic syndrome. *Pediatr Res* 2002; **52**: 307-313
17 **Bub A**, Watzl B, Blockhaus M, Briviba K, Liegibel U, Muller H, Pool-Zobel BL, Rechkemmer G. Fruit juice consumption modulates antioxidative status, immune status and DNA damage. *J Nutr Biochem* 2003; **14**: 90-98
18 **Meydani SN**, Wu D, Santos MS, Hayek MG. Antioxidants and immune response in aged persons: overview of present evidence. *Am J Clin Nutr* 1995; **62**(6 Suppl): 1462S-1476S
19 **Mosmann TR**, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; **17**: 138-146
20 **Exon JH**, Magnuson BA, South EH, Hendrix K. Dietary quercetin, immune functions and colonic carcinogenesis in rats. *Immunopharmacol Immunotoxicol* 1998; **20**: 173-190
21 **Falchetti R**, Fuggetta MP, Lanzilli G, Tricarico M, Ravagnan G. Effects of resveratrol on human immune cell function. *Life Sci* 2001; **21**: 81-96

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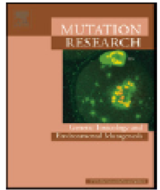

Naturally occurring chlorophyll derivatives inhibit aflatoxin B_1-DNA adduct formation in hepatoma cells

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ABSTRACT

The inhibitory effects of four chlorophyll derivatives (chlorophyllide [Chlide] a and b and pheophorbide [Pho] a and b) on aflatoxin B_1 (AFB_1)-DNA adduct formation, and on the modulation of hepatic glutathione *S*-transferase (GST) were evaluated in murine hepatoma (Hepa-1) cells. Enzyme-linked immunosorbent assay showed that pretreatment with Chlide or Pho significantly reduced the formation of AFB_1-DNA adducts, and that Pho was the most potent inhibitor. However, wash-out prior to adding AFB_1 totally eliminated inhibition by Childe and partially eliminated inhibition by Pho, indicating that the inhibitory effect of Chlide, and to some extent Pho, was mediated through direct trapping of AFB_1. Furthermore, spectrophotometric analysis showed that Pho treatment could increase GST activity in Hepa-1 cells. These observations indicate that the chlorophyll derivatives studied may attenuate AFB_1-induced DNA damage in the Hepa-1 cell by direct trapping of AFB_1. Pho provided additional protection not only by direct trapping, but also by increasing GST activity against hepatic AFB_1 metabolites.

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1. Introduction

Epidemiological studies have demonstrated that high consumption of fruits and vegetables is associated with reduction of cancer risk in humans [1]. The beneficial effects of fruits and vegetables have been partly attributed to the presence of numerous phytochemicals. However, many of these phytochemicals elicit chemopreventative effects in experimental animals only at doses far above the concentrations commonly encountered in the human diet. One promising exception may be chlorophyll and chlorophyll-related chemicals.

Chlorophylls are naturally catabolized into two related chemicals, chlorophyllide and pheophorbide (Fig. 1). The chlorophylls found in green vegetables are made of a porphyrin ring to which is attached a long-chain phytol tail, and in which a Mg atom is chelated. Removal of the phytol tail from chlorophyll forms chlorophyllide (Chlide), and the removal of both the phytol tail and the chelated Mg atom forms pheophorbide (Pho). The chlorophylls chlorophyll a and chlorophyll b vary slightly in the chemical structure of their porphyrin ring and are converted into chlorophyllide a (Chlide a) and pheophorbide a (Pho a) or chlorophyllide b (Chlide b) and pheophorbide b (Pho b), respectively. These naturally occurring chlorophyll derivatives are abundant in green vegetables, but only a few studies have explored their chemopreventative properties [1–4].

In contrast, extensive studies have been done with Chlorophyllin (Chllin). Chllin is a commercially prepared, water-soluble, sodium–copper salt derivative of chlorophyll sold under the trade name Derifil. Chllin has been shown to be antimutagenic [5] and anticarcinogenic [6] when tested against various carcinogens. In particular Chllin has been shown to protect against the cancer-causing aflatoxins [7]. The mechanism of action has been demonstrated to involve trapping carcinogens via binding of the planar ring structures of the carcinogens to the planar ring structure in Chllin [8].

Aflatoxins are toxic metabolites produced by certain fungi. Aflatoxins regularly contaminate foods such as maize, peanuts, and fermented soybeans. The problem was first recognized in 1960 when

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Fig. 1. Chlorophylls and their derivatives. Chlorophylls a and b are converted into their respective derivatives by dephytylation into chlorophyllide and subsequent magnesium dechelation into pheophorbide.

there was severe outbreak of a disease referred as "Turkey 'X' Disease" in the U.K., in which over 100,000 turkey poults died. Aflatoxin B_1 (AFB_1) is the most prevalent aflatoxin. Studies have shown that concurrent infection with the Hepatitis B virus (HBV) during aflatoxin exposure increases the risk of hepatocellular carcinoma (HCC) in developing countries [9], particularly in Africa and Southeast Asia. Aflatoxin exposure and HBV infection are cosidered to be the two major risk factors for HCC. Although HBV can be controlled by vaccination, few interventions for aflatoxin exposure are available.

In animals, aflatoxin is metabolized through a number of competing pathways. In one pathway, AFB_1 has been shown to be activated by cytochrome P4503A4 (CYP3A4) into an epoxide, AFB_1 8,9-epoxide [10]. This highly reactive epoxide is known to form a covalent adduct with guanine yielding AFB_1-N^7-guanine (the major AFB_1-DNA adduct in the liver) [11]. Studies have shown that AFB_1-N^7-guanine causes a GC→TA transversion at a hotspot centered around codon 249 in the p53 gene [12], and that this p53-inactivating mutation is frequently found in HCC patients [13]. The adduct AFB_1-N^7-guanine is therefore used as a biomarker for aflatoxin poisoning, and it has been observed that elevated levels of this adduct are associated with an increased risk of liver cancer [7].

A major metabolic pathway detoxifying AFB_1 involves the glutathione *S*-transferase (GST) enzymes. GST enzymes conjugate AFB_1 8,9-epoxide with glutathione, preventing the epoxide from forming an adduct with DNA and facilitating the clearance of the bound epoxide from the body. In mice, the constitutive activity of alpha-class liver GSTs is high enough to protect mice from AFB_1 exposure, whereas in rats the constitutive GST activity is not great enough to protect them from AFB_1 exposure [14]. Experimentally feeding rats phytochemicals can boost hepatic GST activity and protect against AFB_1 exposure [15]. It has similarly been observed in primates that *Macaca fascicularis* has a naturally high GST activity against AFB_1, whereas humans do not [16].

In this study we challenged murine Hepa-1 hepatoma cell cultures with AFB_1 and measured the protective effects of the chlorophyll compounds Chllin, Chlide a, Chlide b, Pho a, and Pho b. AFB_1-DNA adduct formation was used as a measure. A wash-out experiment was used to see if the chlorophyll compounds produced their protective effect by conjugating with AFB_1. GST activity was measured to see if the protective effects correlated to an increase in GST activity. To our knowledge this is the first test of Chlide or Pho chemoprevention against AFB_1 toxicity. This study will help elucidate the basis of epidemiological observations of dietary cancer prevention in humans as well as explore the mechanism of action of these chlorophyll derivatives.

2. Materials and methods

2.1. Chlorophyll derivatives

Chlorophyll derivatives were prepared from spinach purchased in a local market in Taipei, Taiwan. They were prepared as previously described [17]. Briefly, chlorophylls a and b were extracted from the spinach was purchase from a local supplier, washed with cold water, and quickly freeze-dried the sample with liquid nitrogen and grind it into powder with pestle and stored at −70 °C until extraction. Grind and extract total pigment with 80% acetone; centrifuge the crude extract at 1500 × *g* for 5 min; and keep the supernatant and discard the pellet. Subsequently chlorophyll a and b were purified by liquid chromatography using a combination of ion-exchange and size exclusion chromatography with a CM-Sepharose CL-6B column. Analyses of chromatography fractions were performed by measuring the absorbance at 663.6 and 646.6 nm, which are the major absorption peaks of chlorophyll a and b. Chlorophyll a and b were dephytylated to Chlide a and b, respectively, by chlorophyllase isolated from the leaf of *Ficus macrocarpa*. The Chlide a and b were further Mg-dechelated to form Pho a and b by acidification with acetic acid, and allowed to stand for 2 min. All the samples were then dried under helium and stored at −70 °C for later use. Chlide and Pho, being more polar than chlorophyll, could be dissolved in phosphate-buffered saline (PBS) for use.

The purity of the chlorophyll derivatives was higher than 95%, as determined by reversed-phase high-performance liquid chromatography (RP-HPLC). Analytical separations were performed on a 5 μm Spherisorb ODS-2 column (25 cm × 0.4 cm, C_{18}). The chlorophyll derivatives were detected by fluorescence detection (excitation and emission wavelengths at 440 and 660 nm) and eluted using a linear gradient from solvent A (80% methanol in 1 M ammonium acetate) to solvent B (80% methanol in 1 M acetone) (Fig. 2) [18]. Chllin was from Sigma Chemical Co. (St. Louis, MO).

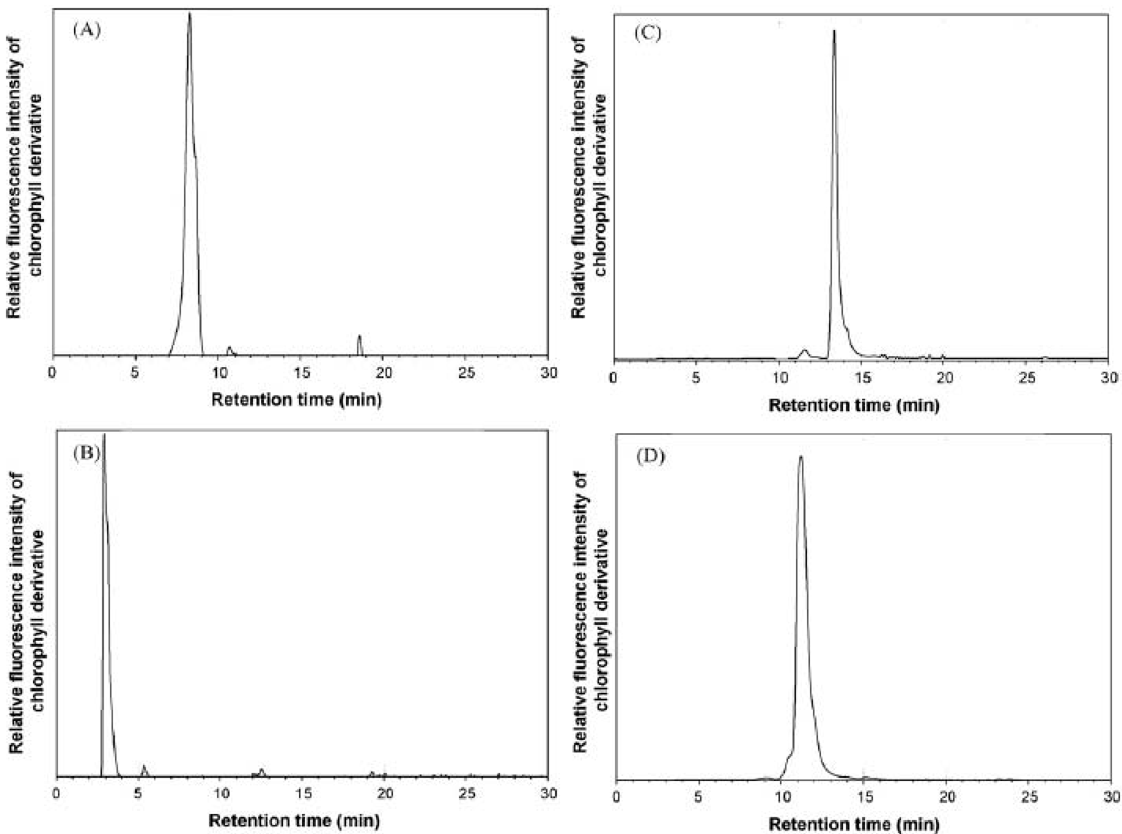


Fig. 2. Elution profile of the chlorophyll derivatives by RP-HPLC. Chlorophyll derivatives (chlorophyllide a (A); chlorophyllide b (B); pheophorbide a (C); and pheophorbide b (D)) were separated on a 5 μm Spherisorb ODS-2 column (25 cm × 0.4 cm, C_{18}), eluted with solvent A (80% methanol in 1 M ammonium acetate) and solvent B (80% methanol in 1 M acetone) by a linear gradient, and detected by a fluorescence detector (excitation and emission at 440 and 660 nm, respectively). All compounds prepared for this study showed greater than 95% purity.

2.2. Cell culture

In this study we used Hepa-1, an immortalized murine hepatoma cell line (Hepa-1c1c7) obtained from the Bioresource Collection and Research Centre (Taiwan). Although mice have been observed to be resistant to AFB_1 toxicity, the Hepa-1 cell line is highly sensitive to AFB_1 [19]. Cells were maintained by subculturing twice a week in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum and incubating at 37 °C in a humidified atmosphere of 5% CO_2/95% air. For use in experiments, cells were harvested from continuous culture and adjusted to the appropriate cell density after counting on a hemocytometer.

In a control experiment to rule out cytotoxicity, Hepa-1 cells were cultured with the highest concentration of chlorophyll derivatives used (50 μM) and all the concentrations of AFB_1 used in later experiments. The media change protocol was the same as that used in AFB_1-DNA adducts analysis described below. Cell viability was determined at 96 h using an MTS assay kit (Promega, Madison, WI) following the manufacturer's instructions [20].

2.3. Analysis of aflatoxin B_1-DNA adducts

The effect of chlorophyll derivatives on AFB_1-DNA adduct formation was studied by treating Hepa-1 cells with different concentrations of AFB_1 and chlorophyll derivatives. Briefly, 1×10^6 Hepa-1 cells were cultured for 96 h with daily media changes. Cells received two PBS washes during each media change. The 0 and 24 h media changes contained different concentrations (0, 5, 20, or 50 μM) of chlorophyll derivatives (Chlide a, Chlide b, Pho a, Pho b, or Chllin). The 48 and 72 h media changes contained the chlorophyll derivatives and AFB_1 (0, 5, or 10 ng/ml). The wash-out variation of this experiment used media containing only AFB_1 during the 48 and 72 h media changes. In order to measure DNA damage after AFB_1 treatment, DNA was purified at 96 h from treated Hepa-1 cells using a PUREGENE® DNA Isolation Kit (Gentra Systems, Minneapolis, MN).

The levels of AFB_1-DNA adducts were then measured by competitive ELISA using antibody 6A10 as described previously [21]. Briefly, the ELISA used Immulon 2 plates (Dynatech Laboratories, Chantilly, VA) coated with 5 ng of imidazole ring-opened AFB_1-DNA in PBS by drying overnight at 37 °C. The test solutions contained unbound AFB_1-DNA and antibody. Goat anti-mouse IgG alkaline phosphatase (1:1500) and then *p*-nitrophenyl phosphate (1 mg/ml in 1 M diethanolamine, pH 8.6) was added to the DNA. After 90 min incubation at 37 °C, absorbance at 405 nm was read on a Bio-Tek microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

For the test samples, 25 μg denatured Hepa-1 DNA in 50 μl hydration solution was mixed with 50 μl diluted antibody before being added to the wells. The level of AFB_1-DNA in the test samples was quantitated relative to a standard curve based on known concentrations of AFB_1-DNA.

For the standard curve, highly modified imidazole ring-opened AFB_1-DNA was serially diluted with nonmodified denatured calf thymus DNA such that 50 μl contained from 0 to 1000 fmol adduct and 50 μg DNA. These samples were mixed with an equal volume of diluted 6A10 antibody (50 μl, diluted 1:1.25 × 10^6), added to the wells, and measured by competitive ELISA.

2.4. Analysis of glutathione S-transferase (GST) activity

GST activity was measured with a GST assay kit (Calbiochem, Bad Soden, Germany) using glutathione (GSH) and 1-chloro-2,4-dinitrobenze (CDNB) as substrates [22]. Briefly, CDNB–GSH conjugate formation (GST activity) was recorded continuously in potassium phosphate buffer (pH 6.5) containing cell lysates, GSH, and CNDB using a spectrophotometer set at 340 nm. The slopes (ΔA_{340}/min) of the sample and blank were recorded over a 5-min period, and the extinction coefficient of the CDNB–GSH conjugate was 9.6 $mM^{-1} cm^{-1}$. The activity was expressed as Δ concentration of CDNB–GSH conjugate/mg protein.

2.5. Statistical analysis

All values were expressed as means ± S.D. Data were analyzed using SPSS software (Version 10.0). Statistical significance was determined using one way ANOVA followed by Scheffe's *post hoc* multiple comparison test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity

We first tested our experimental system to see if any of the components caused cytotoxicity when measured by MTS assay. Treatment of Hepa-1 cells with the maximum dose (50 μM) of chlorophyll derivatives alone showed no significant decrease in cell survival. Likewise, when treated with either AFB_1 alone or in combination with chlorophyll derivatives, no significant decrease in cell survival was observed.

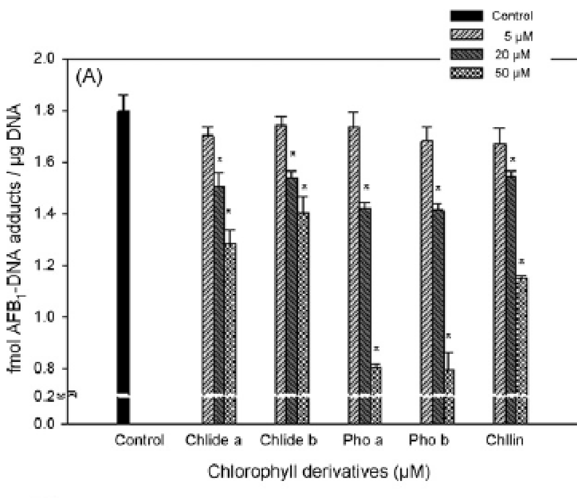
3.2. Effect of chlorophyll derivatives on formation of AFB_1-DNA adducts in Hepa-1 cells

As a biomarker for mutagenicity, AFB_1-DNA adducts were measured in a competitive ELISA with a monoclonal antibody designed to specifically bind AFB_1-DNA adducts [21]. Fig. 3A shows the amount of AFB_1-DNA adduct formed after treatment with 5 ng/ml AFB_1 in the presence of 0–50 μM of each chlorophyll derivative. All chlorophyll derivatives increasingly inhibited AFB_1-DNA adduct formation as the dose was increased. At high concentrations, all chlorophyll derivatives showed statistically significant inhibition. Pho a and b produced the greatest inhibition among the chlorophyll derivatives.

Fig. 3B shows the same experiment as above using 10 ng/ml AFB_1. Note that the baseline with 0 μM of each chlorophyll derivative has a higher amount of AFB_1-DNA adduct formation than was seen with 5 ng/ml AFB_1, consistent with an AFB_1-dose-dependent formation of adducts. Again, increasing concentrations of chlorophyll derivatives showed increasing and statistically significant inhibition of AFB_1-DNA adduct formation with Pho a and Pho b among the most potent inhibitors at high concentration.

3.3. Effect of wash-out on formation of AFB_1-DNA adducts in Hepa-1 cells

All or part of the inhibition of formation of AFB_1-DNA adducts in Hepa-1 cells seen after chlorophyll derivatives treatment may be due to direct binding of AFB1 by the derivatives, thus preventing AFB_1 from being metabolized into AFB_1 8,9-epoxide and forming DNA adducts. In order to address this possibility, a wash-out experiment was performed in which the cells were pretreated



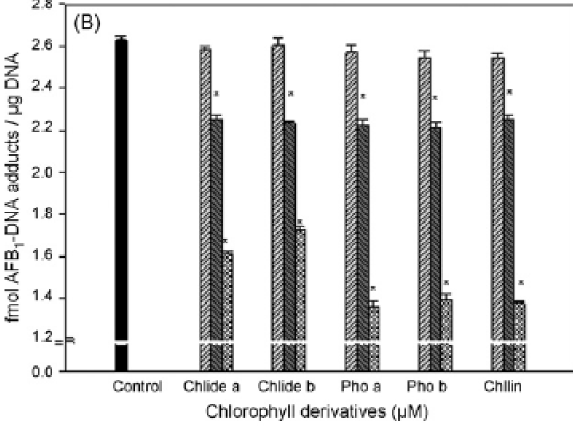
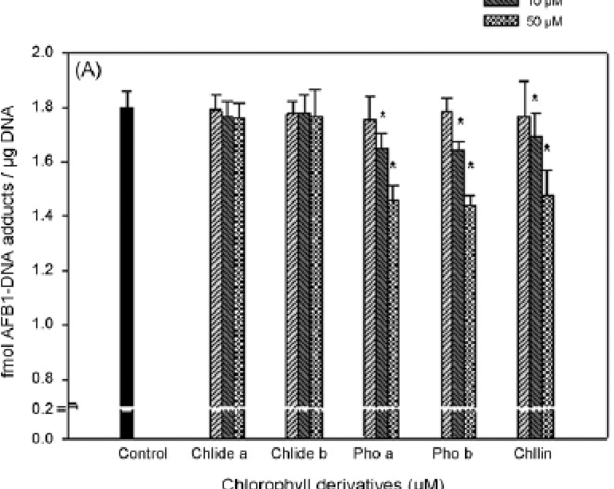


Fig. 3. Effect of pretreatment with chlorophyll derivatives before challenge with 5 ng/ml (A) or 10 ng/ml (B) AFB_1. AFB_1-DNA adduct formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a biomarker for AFB_1 carcinogenic potential. Results are expressed as the mean ± S.D. Note that histograms (A) and (B) do not use the same scale *y*-axis. (*) Significant difference (P< 0.05) from control value.



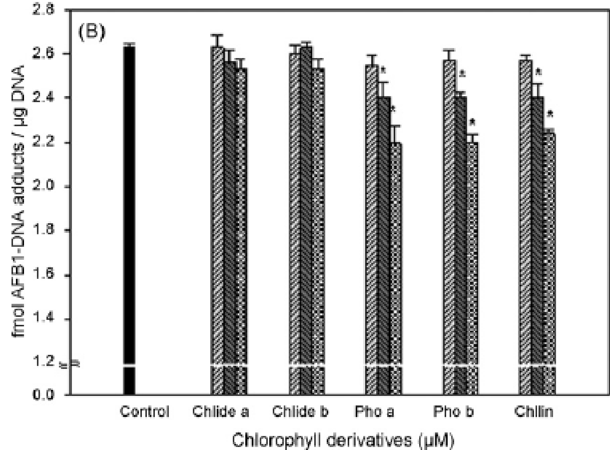


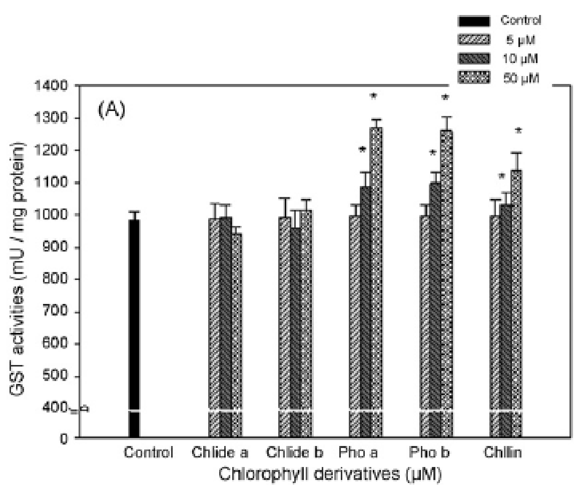
Fig. 4. Effect of pretreatment with followed by wash-out of chlorophyll derivatives before challenge with 5 ng/ml (A) or 10 ng/ml (B) AFB_1. AFB_1-DNA adduct formation in extracted Hepa-1 cell DNA was measured using ELISA assay as a proxy for AFB_1 carcinogenic potential. Results are expressed as the mean ± S.D. Note that histograms (A) and (B) do not use the same scale *y*-axis. (*) Significant difference (P< 0.05) from control value.

with chlorophyll derivatives during the first two media changes, but only AFB_1 and no chlorophyll derivatives were present in last two the media changes. We reasoned that if the chlorophyll derivatives inhibited AFB_1 adduct formation by directly binding the AFB_1, then there should be no inhibition of AFB_1 if there was no chlorophyll derivate present in the media during treatment with AFB_1.

Fig. 4A shows the results of the wash-out experiments with 5 ng/ml AFB_1. Compare Fig. 4A to the parallel experiment in Fig. 3A and note that in the cultures receiving no chlorophyll derivative treatments (0 μM), the level of AFB_1-DNA adduct formation is the same; this indicates that the two experimental data sets are in agreement. All chlorophyll derivatives at all concentrations inhibited AFB_1-DNA adduct formation less in the wash-out experiment. Most dramatically, Chlide a and b no longer inhibited AFB_1-DNA adduct formation in the wash-out experiment. Pho a, Pho b and Chllin all had reduced inhibition of AFB_1-DNA adduct formation in the wash-out experiment, but could still significantly inhibit adduct formation. Fig. 4B shows similar results when using a concentration of 10 ng/ml AFB_1.

3.4. *Effect of chlorophyll derivatives on GST activity in Hepa-1 cells*

To explore the possibility of an indirect protective effect of chlorophyll derivatives on AFB_1-induced DNA damage, the effect of chlorophyll derivatives on GST activity in AFB_1-treated Hepa-1 cells was studied (Fig. 5). Chlide a and b did not show any statistically significant difference in GST activity in AFB_1-treated (at either 5 or 10 ng/ml) Hepa-1 cells. Pho a, Pho b and Chllin treatment had significant dose-dependent increases in GST activity.



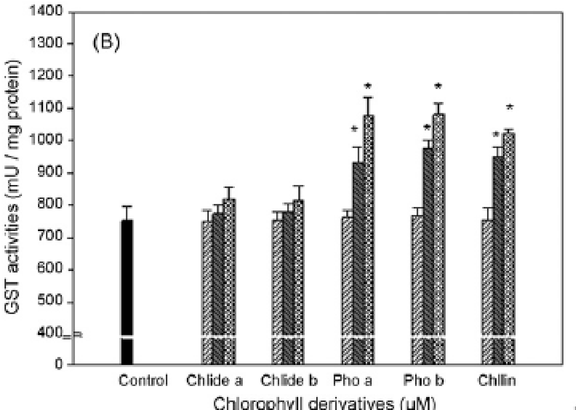


Fig. 5. Effect of chlorophyll derivatives on GST activity in AFB_1-induced Hepa-1 cells. Cells were first treated with various derivatives for 48 h and then treated with 5 ng/ml (A) or 10 ng/ml (B) AFB_1 for another 48 h. GST activity was evaluated using a commercial kit. Results are expressed as the mean ± S.D. (*) Significant difference ($P < 0.05$) from control value.

4. Discussion

In this study we looked at the protective effects of chlorophyll derivatives against AFB_1-DNA adduct formation. We found a significant inhibition of AFB_1-DNA adduct formation. We further showed that the wash-out experiment partially or completely eliminated this inhibitory effect. Finally, we examined the effects of the test compounds on GST activation and found that Pho and Chllin could significantly increase GST activity.

Pho a can be used to kill cells through photodynamic therapy, where a light source such as a laser is used to stimulate free radical production by Pho a, ultimately leading to growth arrest and apoptosis [23,24]. Because chlorophyll compounds can become cytotoxic in certain situations, we first tested for cytotoxicity and found that none of the treatments used in the study caused cell death.

Levels of AFB_1-DNA adducts have been positively associated with risk of liver cancer and have been used as a biomarker of AFB_1 exposure [25]. By measuring AFB_1-DNA adduct formation we were able to observe protection against adduct formation by chlorophyll derivatives in a dose-dependent manner. The degree of protection was greatest for Pho and least for Chlide.

Numerous *in vitro* studies have indicated that chlorophyll derivatives attenuate chemical genotoxicity by forming a molecular complex with promutagens [8,26–28], which may involve strong chlorophyll–AFB_1 interaction *via* their planar unsaturated cyclic rings [27]. The complete elimination of the Chlide inhibitory effect after the wash-out experiment shows that the effects of Chlide occur in the culture media and not in the cells. The best explanation for its activity is that Chlide is directly binding to and neutralizing AFB_1. The fact that the effects of Chlide are dose-responsive also supports such a conclusion.

That Chllin and Pho also showed a reduction of ability to inhibit AFB_1-DNA adduct formation in the wash-out experiment means that part of their effects are related to their presence in the cell culture media. The simple explanation that Chllin and Pho also bind and neutralize AFB_1 is reasonable. That Chllin and Pho retained activity after wash-out was surprising. Hypothesizing that some Pho may have been absorbed into the cells, we performed a control experiment (data not shown) in which Hepa-1 cells were treated with the test compounds, Chlide a and b and Pho a and b, for 48 h then thoroughly washed free of medium and tested for presence of these chlorophyll derivatives in the cells by HPLC analysis of ultra-sonicated cell lysates; Pho a and b could be found in the cell lysates whereas very little Chlide a and b could be found. Thus, in the wash-out experiment, Pho was retained between media changes sequestered in the cells and was available to bind to AFB_1 entering the cells when the cells were challenged with AFB_1

However, it is also possible that, in addition to directly binding AFB_1, Chllin and Pho (but not Chlide) interact with and change the Hepa-1 cells, increasing cellular resistance to AFB_1-DNA adduct formation. Thus, part of the protection provided by Chllin and Pho would have been via direct binding of AFB_1 and part of the protection would have been provided by stimulation of cellular defenses. Such a hypothesis also fits the wash-out data; after the pretreatment media containing Chllin or Pho was removed and the AFB_1-containing media was added, the cells could still have been resistant to AFB_1 because the cells themselves had become resistant to AFB_1 due to a dose-dependent stimulation of cellular defenses.

This mechanistic hypothesis is attractive because it would also explain why there is a difference in the magnitude of the protective inhibitory effect of Chlide versus Pho or Chllin. Because the chlorophyll derivatives have similar molecular structures and were present in the same molarities, a simple binding reaction would be expected to remove and inactivate similar quantities of AFB_1. The larger inhibitory effects of Pho and Chllin could be the additive result of their stimulation of cellular defenses plus their AFB_1 binding. If their putative cell stimulatory effects were blocked and only their ability to bind AFB_1 remained, we would expect that the degree of inhibition might be close to that of Chlide.

In order to look for stimulation of cellular defenses, we measured the ability of the chlorophyll derivatives to increase GST activity. Chllin and Pho were able to significantly increase GST activity in a dose-dependent manner in Hepa-1 cells, while Chlide could not. Thus, it seems reasonable to conclude that the inhibition of AFB_1-DNA adduct formation by Chlide a and Chlide b was due to direct molecular trapping of AFB_1, and that the greater inhibition of AFB_1-DNA adduct formation by Chllin, Pho a and Pho b was due to both direct molecular trapping of AFB_1 and stimulation of cellular defenses.

Little has been published about the absorption of chlorophyll derivatives into cells, however, some data is available about the absorption of Pho a. In general, it appears that passive diffusion of Pho a into cells is counteracted by active transport of Pho a out of cells by ATP-binding cassette (ABC) transporters such as the breast cancer resistance protein (ABCG2) [29]. Tumor cells have been noted to preferentially absorb Pho a compared to normal cells [30–32] and to concentrate Pho a in the mitochondria [33,34]. In contrast, drug-resistant cancer cell lines typically have increased expression of the ABC transporters. AFB_1 penetrance into cells is similarly affected by ABC transporters [35]. The fact that the Hepa-1 cell line used in this experiment is highly sensitive to AFB_1 suggests that Hepa-1 cells have low ABC transporter expression. This agrees with our data showing that the Hepa-1 cells absorbed Pho. Whether or not any absorbed Pho or Chllin would have passively diffused out of the cells and diluted into the fresh AFB_1-containing medium during the washout experiment remains to be investigated.

Fahey et al. [36] directly tested Chllin against murine hepatoma cells *in vitro* and showed that Chllin induces the phase II enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1). Likewise, Singh et al. [37] showed that short-term oral administration of Chllin increases the levels of hepatic GST in lactating mice and suckling pups. These *in vivo* and *in vitro* results parallel our *in vitro* results. In contrast Simonich et al. [38] also tested the ability of Chllin and chlorophyll to prevent AFB_1 toxicity *in vivo*. Rats given dietary Chllin and chlorophyll and then challenged with AFB_1 have reduced AFB_1-adduct formation, but do not show a significant effect on the phase II enzymes GST and NQO1 [42]. Thus, the *in vitro* results presented here need to be weighed against the possibility that much of the dietary chlorophyll compounds are kept from entering the body by ABC transporters in the intestines and that the majority of chlorophyll compounds' interaction with dietary AFB_1 *in vivo* may take place in the intestinal lumen through direct trapping.

In conclusion, we have provided evidence that chlorophyll derivatives can reduce AFB_1-DNA adduct formation *in vitro*. Diets rich in chlorophyll may prevent the development of hepatocellular carcinoma. We also provide evidence that the mechanisms and intensity of protective effects may be dependent on the particular chlorophyll derivative used.

5. Conflict of interest

None.

Acknowledgments

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References

[1] G. Block, B. Patterson, A. Subar, Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence, Nutr. Cancer 18 (1992) 1–29.
[2] T. Negishi, H. Rai, H. Hayatsu, Antigenotoxic activity of natural chlorophylls, Mutat. Res. 376 (1997) 97–100.
[3] C.N. Lai, M.A. Butler, T.S. Matney, Antimutagenic activities of common vegetables and their chlorophyll content, Mutat. Res. 77 (1980) 245–250.
[4] U. Harttig, G.S. Bailey, Chemoprotection by natural chlorophylls in vivo: inhibition of dibenzo[a,l]pyrene-DNA adducts in rainbow trout liver, Carcinogenesis 19 (1998) 1323–1326.
[5] R.H. Dashwood, V. Breinholt, G.S. Bailey, Chemopreventive properties of chlorophyllin: inhibition of aflatoxin B1 (AFB1)-DNA binding in vivo and anti-mutagenic activity against AFB1 and two heterocyclic amines in the *Salmonella* mutagenicity assay, Carcinogenesis 12 (1991) 939–942.
[6] V. Breinholt, D. Arbogast, P. Loveland, C. Pereira, R. Dashwood, J. Hendricks, G. Bailey, Chlorophyllin chemoprevention in trout initiated by aflatoxin B(1) bath treatment: an evaluation of reduced bioavailability vs. target organ protective mechanisms, Toxicol. Appl. Pharmacol. 158 (1999) 141–151.
[7] P.A. Egner, J.B. Wang, Y.R. Zhu, B.C. Zhang, Y. Wu, Q.N. Zhang, G.S. Qian, S.Y. Kuang, S.J. Gange, L.P. Jacobson, K.J. Helzlsouer, G.S. Bailey, J.D. Groopman, T.W. Kensler, Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 14601–14606.
[8] S. Arimoto, S. Fukuoka, C. Itome, H. Nakano, H. Rai, H. Hayatsu, Binding of polycyclic planar mutagens to chlorophyllin resulting in inhibition of the mutagenic activity, Mutat. Res. 287 (1993) 293–305.
[9] J.H. Williams, T.D. Phillips, P.E. Jolly, J.K. Stiles, C.M. Jolly, D. Aggarwal, Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions, Am. J. Clin. Nutr. 80 (2004) 1106–1122.
[10] F.P. Guengerich, W.W. Johnson, T. Shimada, Y.F. Ueng, H. Yamazaki, S. Langouet, Activation and detoxication of aflatoxin B1, Mutat. Res. 402 (1998) 121–128.
[11] R.G. Croy, J.M. Essigmann, V.N. Reinhold, G.N. Wogan, Identification of the principal aflatoxin B1-DNA adduct formed in vivo in rat liver, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 1745–1749.
[12] P.L. Foster, E. Eisenstadt, J.H. Miller, Base substitution mutations induced by metabolically activated aflatoxin B1, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 2695–2698.
[13] P.E. Jackson, S.Y. Kuang, J.B. Wang, P.T. Strickland, A. Munoz, T.W. Kensler, G.S. Qian, J.D. Groopman, Prospective detection of codon 249 mutations in plasma of hepatocellular carcinoma patients, Carcinogenesis 24 (2003) 1657–1663.
[14] J.D. Hayes, D.J. Judah, G.E. Neal, T. Nguyen, Molecular cloning and heterologous expression of a cDNA encoding a mouse glutathione *S*-transferase Yc subunit possessing high catalytic activity for aflatoxin B1-8,9-epoxide, Biochem. J. 285 (1) (1992) 173–180.
[15] V.P. Kelly, E.M. Ellis, M.M. Manson, S.A. Chanas, G.J. Moffat, R. McLeod, D.J. Judah, G.E. Neal, J.D. Hayes, Chemoprevention of aflatoxin B1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of aflatoxin B1-aldehyde reductase, the glutathione *S*-transferase A5 and P1 subunits, and NAD(P)H:quinone oxidoreductase in rat liver, Cancer Res. 60 (2000) 957–969.
[16] C. Wang, T.K. Bammler, Y. Guo, E.J. Kelly, D.L. Eaton, Mu-class, GSTs are responsible for aflatoxin B(1)-8,9-epoxide-conjugating activity in the nonhuman primate *Macaca fascicularis* liver, Toxicol. Sci. 56 (2000) 26–36.
[17] C.M. Yang, K.W. Chang, M.H. Yin, H.M. Huang, Methods for the determination of the chlorophylls and their derivatives, Taiwania 43 (1998) 116–122.
[18] L. Almela, J.A. Fernandez-Lopez, M.J. Roca, High-performance liquid chromatographic screening of chlorophyll derivatives produced during fruit storage, J. Chromatogr. A 870 (2000) 483–489.
[19] S.O. Karenlampi, Mechanism of cytotoxicity of aflatoxin B1: role of cytochrome P1-450, Biochem. Biophys. Res. Commun. 145 (1987) 854–860.
[20] A.H. Cory, T.C. Owen, J.A. Barltrop, J.G. Cory, Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture, Cancer Commun. 3 (1991) 207–212.
[21] L.L. Hsieh, S.W. Hsu, D.S. Chen, R.M. Santella, Immunological detection of aflatoxin B1-DNA adducts formed in vivo, Cancer Res. 48 (1988) 6328–6331.
[22] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[23] R.F. Haseloff, B. Ebert, B. Roeder, Generation of free radicals by photoexcitation of pheophorbide alpha, haematoporphyrin and protoporphyrin, J. Photochem. Photobiol. B 3 (1989) 593–602.
[24] W.Y. Lee, D.S. Lim, S.H. Ko, Y.J. Park, K.S. Ryu, M.Y. Ahn, Y.R. Kim, D.W. Lee, C.W. Cho, Photoactivation of pheophorbide a induces a mitochondrial-mediated apoptosis in Jurkat leukaemia cells, J. Photochem. Photobiol. B 75 (2004) 119–126.

[25] J.M. Essigmann, R.G. Croy, A.M. Nadzan, W.F. Busby Jr., V.N. Reinhold, G. Buchi, G.N. Wogan, Structural identification of the major DNA adduct formed by aflatoxin B1 in vitro, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 1870–1874.

[26] N. Tachino, D. Guo, W.M. Dashwood, S. Yamane, R. Larsen, R. Dashwood, Mechanisms of the in vitro antimutagenic action of chlorophyllin against benzo[a]pyrene: studies of enzyme inhibition, molecular complex formation and degradation of the ultimate carcinogen, Mutat. Res. 308 (1994) 191–203.

[27] V. Breinholt, M. Schimerlik, R. Dashwood, G. Bailey, Mechanisms of chlorophyllin anticarcinogenesis against aflatoxin B1: complex formation with the carcinogen, Chem. Res. Toxicol. 8 (1995) 506–514.

[28] T. Negishi, S. Arimoto, C. Nishizaki, H. Hayatsu, Inhibitory effect of chlorophyll on the genotoxicity of 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), Carcinogenesis 10 (1989) 145–149.

[29] J.W. Jonker, M. Buitelaar, E. Wagenaar, M.A. Van Der Valk, G.L. Scheffer, R.J. Scheper, T. Plosch, F. Kuipers, R.P. Elferink, H. Rosing, J.H. Beijnen, A.H. Schinkel, The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15649–15654.

[30] H. Hayashi, S. Nakajima, N. Samejima, Basic study for cancer therapy with porphyrin derivatives and pheophorbide derivatives, Hokkaido Igaku Zasshi 60 (1985) 391–403.

[31] S. Chernomorsky, A. Segelman, R.D. Poretz, Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth, Teratogen. Carcinogen. Mutagen. 19 (1999) 313–322.

[32] A. Hajri, S. Wack, C. Meyer, M.K. Smith, C. Leberquier, M. Kedinger, M. Aprahamian, In vitro and in vivo efficacy of photofrin and pheophorbide a, a bacteriochlorin, in photodynamic therapy of colonic cancer cells, Photochem. Photobiol. 75 (2002) 140–148.

[33] P.M. Tang, J.Y. Chan, S.W. Au, S.K. Kong, S.K. Tsui, M.M. Waye, T.C. Mak, W.P. Fong, K.P. Fung, Pheophorbide a, an active compound isolated from *Scutellaria barbata*, possesses photodynamic activities by inducing apoptosis in human hepatocellular carcinoma, Cancer Biol. Ther. 5 (2006) 1111–1116.

[34] C.S. Kim, C.H. Lee, P.H. Lee, S. Han, Inactivation of mitochondrial electron transport by photosensitization of a pheophorbide a derivative, Mol. Cells 17 (2004) 347–352.

[35] A.E. van Herwaarden, E. Wagenaar, B. Karnekamp, G. Merino, J.W. Jonker, A.H. Schinkel, Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk, Carcinogenesis 27 (2006) 123–130.

[36] J.W. Fahey, K.K. Stephenson, A.T. Dinkova-Kostova, P.A. Egner, T.W. Kensler, P. Talalay, Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes, Carcinogenesis 26 (2005) 1247–1255.

[37] A. Singh, S.P. Singh, R. Bamezai, Modulatory influence of chlorophyllin on the mouse skin papillomagenesis and xenobiotic detoxication system, Carcinogenesis 17 (1996) 1459–1463.

[38] M.T. Simonich, P.A. Egner, B.D. Roebuck, G.A. Orner, C. Jubert, C. Pereira, J.D. Groopman, T.W. Kensler, R.H. Dashwood, D.E. Williams, G.S. Bailey, Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat, Carcinogenesis 28 (2007) 1294–1302.

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Original Article

Consumption of purple sweet potato leaves decreases lipid peroxidation and DNA damage in humans

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Consumption of polyphenols is associated with reduced risk of chronic diseases, possibly via a variety of bio-mechanisms, including antioxidation and anti-inflammation. Purple sweet potato leaves (PSPL) commonly consumed in Asia possess polyphenols. In this study, we aim to investigate antioxidant effect of 200 g/d PSPL containing 902 mg polyphenols in a clinical trial. This randomized, crossover clinical study included 16 healthy adults (7 M, 9 F; aged 20-22 y). After a 1-wk run period, subjects were assigned randomly to receive either PSPL or low polyphenol diet (LPD) for 2 wks, followed by a 2-wk washout period before crossing over to the alternate diet. Fasting blood and 24-h urine samples were collected from each subject at day 0, 7 and 14 of each phase. Our data showed PSPL consumption enhanced urinary total phenol excretion by 24.5% at day 14 as compared to day 0, while the LPD decreased total phenol content in plasma and urine by 3.3 and 16.3%, respectively ($p \leq 0.05$). Low-density lipoprotein lag time and glutathione concentration in erythrocytes at day 14 was significantly enhanced by 15.0 and 33.3% by PSPL as compared to day 0, respectively, while their values were not altered by the LPD. Urinary 8-hydoxy-deoxyguanosine (8-OHdG) excretion decreased significantly by PSPL consumptoin by 36.7% at day 7 as compared to day 0, yet unchanged by the LPD ($p \leq 0.05$). In conclusion, our results suggest that polyphenols in 200 g PSPL were bio-available and could enhance antioxidant defense and decrease oxidative stress in young healthy people.

Key Words: purple sweet potato leaves, polyphenols, lipid peroxidation, 8-hydroxydeoxyguanosine, DNA damage

INTRODUCTION

Evidence from epidemiological studies suggested a strong, inverse association between incidence of chronic diseases and intake of plant foods, possibly due to their high nutrient density and low fat contents.[1-5] Thereby, consumption of plant foods has been strongly promoted and promulgated in the dietary guidelines by the public health authorities and regulatory agencies.[6] Nevertheless, contribution of nonessential phytonutrients ubiquitous in plant foods to reduced the risk of health problems via an array of putative mechanism of bioactions, including anti-inflammation, antioxidation, anti-proliferation, and induction of phase II enzymes has been gradually recognized.[7-9] In particular, there is growing interest in polyphenolic compounds because of their prevalence in plants, as well as potent antioxidant activity.[10]

Leaves of sweet potato (*Impoea batatas)* have been consumed commonly in Asian countries and are rich in micronutrients.[11] Because this plant tolerates well against diseases, pest infestation, and flooding,[12] leaves of sweet potato can provide health benefits to people residing in resource poor areas. Like other plant foods, grapes, green tea, onions, these leaves contain polyphenols ranging from 2-14 g/100g dry weight and exhibit antioxidant [13, 14] and anti-mutagenic activity.[15] Recently, we observed in a clinical trial that a 2-wk supplementation of 200 g/d cooked purple sweet potato leaves (PSPL) increased Con A-activated proliferation and IL-2 and -4 secretions in peripheral blood mononuclear cells and elevated lytic activity of NK cells.[16] In other human trial, we also found that 200 g/d PSPL for 2 wks enhanced total phenol content in plasma and LDL resistance against oxidation and decreased urinary 8-hydroxydeguanosine (8-OHdG) in elite basketball players.[17]

While health benefits of polyphenolic compounds could be mediated via a wide spectrum of bioactions, the effect of PSPL incorporated into daily diets on antioxidant defenses and biomarkers of oxidative stress in health individuals remains to be examined. Thus, in this study,

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we aim to investigate whether addition of 200 g/d PSPL to a low polyphenol diet (LPD) for 2 wk can enhance antioxidant defenses and thereby decrease oxidative stress in a cross-over clinical trial. The information gathered from this study is useful for promoting inclusion of sweet potato leaves for health promotion and prevention in resource poor areas.

MATERIALS AND METHODS

Preparation of purple sweet potato leaves

Purple sweet potatoes were planted at the Taoyuan District Agriculture Improvement Station, Taipei Branch, Taiwan, which is 1 hour away from the Taipei Medical University. Fresh PSPL were shipped daily to our metabolic research unit, weighted, washed, stir fried in soy bean oil, and then provided to subjects.

Subjects

Sixteen non-smokers (7 M, 9 F, age: 20-22 yrs, BMI: 20.6-21.4 kg/m^2) in good health condition, based on results from a medical history questionnaire, physical examination, electrocardiogram test, and standard clinical biochemistries. Exclusion criteria included: 1) history of cardiovascular, hepatic, gastrointestinal, and renal disease; 2) alcoholism; 3) use of antibiotics or multi-vitamin and mineral for ≥4 wk prior to the study. Volunteers were asked not to take any vitamin supplement or medication during the whole study period. The study was approved by the Medical Ethical Committee of the Institutional Review Board from Taipei Medical University, and written consent was obtained from each participant.

Study design

A randomized, crossover design was employed in this study. The duration of the whole study was 7 wks, including 1-wk run-in and 2 phases of 2-wk dietary treatment with a 2-wk washout (Figure 1). During the whole study, all subjects were asked to follow a low polyphenol diet (LPD) that excluded berries, apples, pears, citrus fruits, fruit juices, onions, gynura, basil, bok choy, spinach, rabbit milkweed, brassica napus, chocolate, wine, coffee, tea, beans, nuts, soy related products, and most spices.[18] Following the run-in phase, 16 volunteers were assigned randomly to either the PSPL or LPD diet (n = 8). Lunch and dinner meals were provided to all subjects during the study, and were designed by a registered dietitian of the Department of Dietetics in the Taipei Medical University Hospital. They were prepared daily under supervision of the registered dietitian. Meals for one day contained 2000 ± 200 Kcal with 18, 30, and 52% of calories from protein, fat, and carbohydrate, respectively. Typical Chinese lunch and dinner meals consisted of a meat (pork or chicken) dish, a low polyphenols vegetable dish, steamed rice, and a low polyphenols fruit. Two hundred grams of cooked PSPL were divided equally into lunch and dinner meals. In order to ensure good compliance, all participants ate meals in the hospital cafeteria under supervision of the study dietitian. Breakfast was not provided to the subjects in the study, but a list of recommended food items that are low in polyphenols was provided. Further, to monitor compliance to the low polyphenol diet, 3-day dietary records were collected from the subjects every week. Total body fat was assessed using a body fat impedance analyzer at the end of each phase (Inbody 3.0, Biospace, Seoul, Korea).

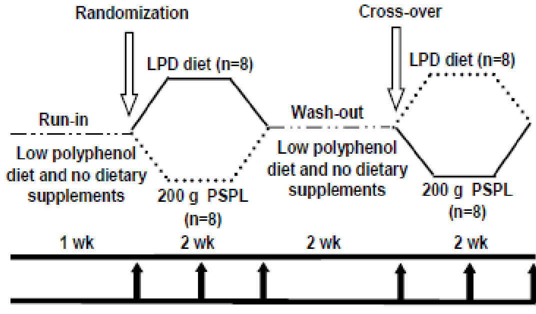


Figure 1. Study design

Sample collection and storage

Six fasted venous blood samples were collected from each subject between 7-9 AM in the study (Fig. 1). Following centrifugation at 1000 x g for 10 min at 4°C, aliquots of plasma samples were snap frozen in liquid nitrogen and stored at -80°C. One aliquot of fresh plasma was used immediately for the LDL oxidation assay on the same day. After washed with ice-cooled saline three times and hemolyzed using ice-cooled distilled water, erythrocytes were stored at -80°C for glutathione (GSH) determination. A total of six 24-h urine samples were collected from each subject on the same day of blood collection. Urine was collected into an amber plastic container and stored at 4°C before it was brought back to the lab. After the volume was recorded, aliquots of urine samples were stored at -20°C for determinations of total phenolic content and 8-OHdG.

Biomarkers of antioxidant defense and oxidative stress

Total phenolic contents in urine and plasma were measured via the Folin-Ciocalteau's reaction, according to the method of Singleton.[19] Results were expressed as gallic acid equivalents (GAE) μmol/L.

Plasma α-Tocopherol was measured using a HPLC method of Milne and Botnen.[20] Total antioxidant status (TAS) in plasma was assessed using a commercial enzymatic assay (Randox, UK). Reduced GSH in erythrocytes was determined using a commercial enzymatic assay (Calbiochem Co., CA, USA). Plasma malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), products of lipid peroxidation, were measured using a commercial enzymatic assay (Calbiochem Co., USA). Urinary 8-OHdG was determined using an ELISA assay (Japan Institute for the Control of Aging, Japan). The resistance of LDL against Cu^{2+}-induced oxidation was determined according to the slightly modified method of Chen et al. [21] Briefly, following a 24-hour dialysis against saline containing Na-EDTA (1 mmol/L), LDL protein was quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, LDL (182 nmol/L) was oxidized by 10 μmol/L $CuSO_4$ in a final volume of 1.0 mL. Formation of conjugated dienes was

monitored by absorbance at 234 nm at 37°C over 6 hour using a UV3000 spectrophotometer (Hitachi, Japan) equipped with a 6-position automated sample changer. The results of the LDL oxidation are expressed as lag time (defined as the intercept at the abscissa in the diene-time plot).

Statistical analysis

All results were reported as mean ± SD. Repeat ANOVA (mix-model) analysis was performed to evaluate changes in parameters in the same dietary group over three time points, student's *t* test was performed to evaluate changes from the d 0 value (D7-D0 and D14-D0) between LPD and PSPL dietary group. p value ≤0.05 was considered significant. The SAS statistical software package (SAS Institute Inc., Cary, NC) was used to perform all statistical analyses.

RESULTS

During the study period, a balanced diet provided to the subjects contained 2000 ± 200 Kcal, 95 ± 10 g protein, 250 ± 25 g carbohydrate, and 69 ± 7 g fat. 200 g cooked PSPL contained 60 Kcal, 6.6 g protein, 1.2 g fat, 9.2 g carbohydrate, 38 mg vitamin C, 170 mg Ca, 40 mg Mg, 902 mg total phenols, and 47.5 mg carotenoids.[22] All 16 subjects completed the 7-wk study and were fully compliant to the LPD, based on the results of dietary records. No significant changes in their BMI, total body fat and clinical biochemistries were observed (Table 1).

PSPL addition to the LPD maintained total phenolic content in plasma while the LPD alone led to a significant 3.3% decrease from 3.59 ± 0.11 to 3.47 ± 0.08 μmol/L at d 14 as compared to that at d 0 (p ≤0.05). Similarly, urinary total phenolic excretion in the LPD group was decreased significantly by 16.3% from 0.49 ± 0.07 μmol/L at d 0 to 0.41 ± 0.08 μmol/L at d 14 (Figure 2). However, urinary total phenol excretion in the PSPL group was significantly augmented by 24.5% at d 14 as compared to that at d 0 (p ≤0.05). Further, the increased plasma total phenolic content from d 14 to d 0 in the PSPL was significantly different from the slightly decreased value in the LPD (p ≤0.05). Similarly, increases in urinary phenolics in the PSPL group at day 7 and 14 as compared day 0 were significantly different from those in the LPD group.

At day 14, PSPL and LPD both decreased plasma α-tocopherol by 31.7 and 15.8% as compared to day 0, respectively (Table 2). Further, the decrease was larger in subjects consuming PSPL than LPD. Erythrocyte GSH status was not significantly altered by the LPD from day 0 to 14, while its concentration was enhanced significantly by PSPL consumption by 33.3 % at day 14 vs. day 0. Further, the increase in erythrocyte GSH from day 0 to day 14 was significantly 72% larger as a result of PSPL intake than the LPD. Total antioxidant status was not significantly altered by the LPD and PSPL from day 0 to 14.

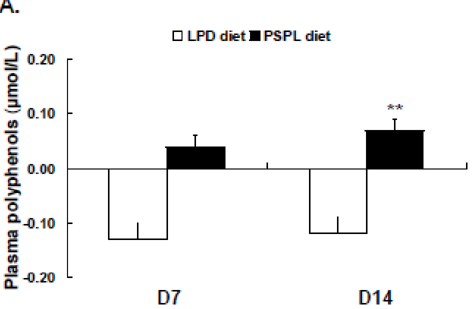
Plasma concentrations of MDA+HNE in the subjects consuming the LPD were significantly decreased by 4.0 % after 1 wk (p ≤0.05). The addition of PSPL into the LPD led to a significant decrease in MDA+HNE by 6.4 % and 5.1% at day 7 and day 14 as compared to that at day 0. However, MDA+HNE concentration at day 14 in the

Table 1. Demographic characteristics and clinical biochemistries of subjects[1]

	Before	After
Age (yr)	20.4±1.8	
Height (cm)	167.8±9.1	
Body weight (kg)	58.7±9.2	59.6±9.2
BMI (kg/m2)	20.8±2.3	21.1±2.2
Body fat (%)	22.3±6.8	23.8±7.0
Creatinine (mg/dL)	0.89±0.15	0.92±0.15
GOT (IU/L)	19.7±6.16	19.0±4.40
GPT (IU/L)	14.80±8.58	13.7±5.94
Triglyceride (mg/dL)	62.2±21.3	73.4±55.8
Cholesterol (mg/dL)	156±24.9	156.0±33.4
HDL-Cholesterol (mg/dL)	58.3±15.8	58.6±17.9
LDL-Cholesterol (mg/dL)	86.1±17.9	82.3±20.2

[1]Resutls were expressed as mean ± SD(n = 16).
**Means significantly differ, tested using pair-t test (p ≤0.05).

A.



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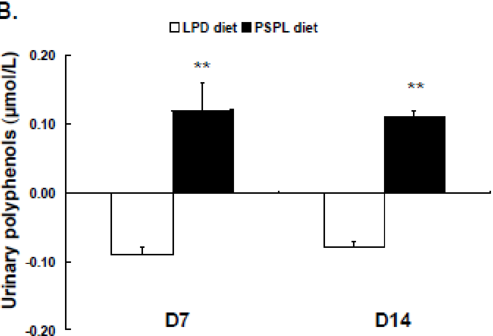

Figure 2. Changes in plasma (A) and urinary (B) polyphenols. Total phenolic content in plasma at day 0 was 3.59 ± 0.11 and 2.87 ± 0.10 μmol/L for LPD and PSPL, respectively, as well as 0.49 ± 0.07 and 0.49 ± 0.09 μmol/L in urine. The results were reported as mean ± SD, n=16. **Means significantly differ between two groups, tested using student's *t* test (p ≤0.05).

Table 2. The status of antioxidants in subjects[†]

	D0	D7	D14
Plasma α-tocopherol (μmol/L)			
LPD	6.2 ± 0.8	7.3 ± 0.8[a]	5.2 ± 0.6[ab]
PSPL	11.4 ± 1.4	9.8 ± 1.4[a]	7.8 ± 0.8[ab]
Erythrocyte GSH (μmol/L)			
LPD	18.5 ± 7.9	18.2 ± 7.0	23.5 ± 6.6
PSPL	25.9 ± 11.7	25.4 ± 10.4	34.5 ± 7.4[a]
Plasma total antioxidant status (mmol/L)			
LPD	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
PSPL	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.2

[†]Resutls were expressed as mean ± SD (n = 16).
[a]Means significantly differ as compared with D0, [b] Means significantly differ between D7 and D14, tested using mix model analysis ($p \leq 0.05$).

Table 3. The status of oxidative stress[†]

	D0	D7	D14
MDA+4HNE (μmol/L)			
LPD	7.5 ± 0.1	7.2 ± 0.2[a]	7.4 ± 0.3[b]
PSPL	7.8 ± 0.3	7.3 ± 0.3[a]	7.4 ± 0.2[a]
8-OHdG (ng/mL)			
LPD	10.2 ± 5.8	9.9 ± 4.8	8.5 ± 3.7
PSPL	8.1 ± 5.6	5.1 ± 4.5[a]	6.9 ± 3.1
LDL lag time (min)			
LPD	73.6 ± 13.9	74.7 ± 10.0	78.1 ± 14.0
PSPL	78.0 ± 12.9	87.1 ± 31.0	89.7 ± 16.5 [a]

[†]Resutls were expressed as mean ± SD (n = 16).
[a] Means significantly differ as compared with D0, [b] Means significantly differ between D7 and D14, tested using mix model analysis ($p \leq 0.05$).

PSPL group was not different from those at day 7. Urinary 8-OHdG, a systematic biomarker of DNA damage, was employed to reveal antioxidant action of constituents in PSPL (Table 3). The LPD did not alter urinary 8-OHdG value from day 0 to day 14 while PSPL consumption significantly decreased urinary 8-OHdG by 36.7% at day 7 as compared to that at day 0 ($p \leq 0.05$). Further, the decrease in 8-OHdG by PSPL consumption from day 0 to day 7 was significantly larger than that by the LPD. The resistance of LDL against Cu^{2+}-induced oxidation significantly increased by 15% after consumption of PSPL for 2 weeks while no significant changes were found in the LPD group. (Table 3).

DISCUSSION

Polyphenols in plant foods may contribute to decreased risk of chronic diseases because of an array of their putative mechanism of actions, i.e., antioxidation, anti-inflammation, and anti-proliferation.[23] Purple sweet potato leaves have been commonly consumed in Asian countries. Since they are rich in various nutrients,[24] incorporation of PSPL into the daily diet may provide benefits in health promotion and prevention. In this study, we observed that the incorporation of 200 g/d PSPL into the LPD for 2 wks enhanced antioxidant defense and decreased oxidative stress in healthy subjects.

Since polyphenols are ubiquitous in plant foods, they are an integral part of our daily diets. It has been estimated that average polyphenol intake probably reaches 1 g/d in people who eat several serving of fruit and vegetables per day.[25] In this study, 902 mg polyphenols from 200 g PSPL added to the LPD provided a comparable quantity of polyphenol intake to the reported value. Consistent with no adverse effect reported in human studies, there were no apparent adverse effects after the consumption of 200 g/d PSPL for 2 wks, according to results of unaltered values of clinical biochemistries, serum creatinine, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic acid transaminase (GPT), triglyceride, and cholesterol, as well as no reported gastrointestinal discomforts (diarrhea, abdominal pain or bloating).

Bioavailability of polyphenols has been documented commonly in humans.[26] Our results showed that 902 mg of polpphenols derived from 200 g/d PSPL consumption enhanced plasma total phenolic content and urinary phenolic excretion suggested bioavailability of polyphenols present in PSPL. However, it is a limitation of this study that we were unable to quantify the bioavailability of individual polyphenols due to unavailability of sophisticated instruments at the time of analysis. Nevertheless, this result was consistent to our unpublished observation that, following 2-hours food deprivation, concentrations of quercetin and caffeic acid in plasma were enhanced in rats fed a PSPL diet. On the other hand, a relatively smaller increase in plasma total phenolic content than changes in urinary phenolic excretion after consuming PSPL for 2 wk might be a result of a rapid clearance of polyphenols because half-lives of polyphenolic compounds are generally shorter than 12 h. [27]

Reactive oxidant species are believed to play an etiological role in pathogenesis of chronic diseases and aging.[28] Well documented antioxidant actions of polyphenols may partially account for decreased risk of oxidative stress-related chronic diseases.[18, 29] Because subjects in this study are healthy and may experience a low degree of oxidative stress, we did not observe the impact of PSPL polyphenols on plasma α-tocopherol status, an outcome consistent to results of our rat study that flavonol quercetin could not prevent decreases in plasma and tissue α-tocopherol in rats fed a vitamin E deplete diet.[30] On the contrary, PSPL polyphenols elevated glutathione status possibly through up-regulating glutathione synthesis and/or preventing glutathione use from consequences of their radical scavenging actions.[31, 32] However, exact mechanism(s) by which PSPL polyphenols modulate glutathione status in humans remain to be investigated. Various total antioxidant capacity assays have been commonly employed to reveal overall antioxidant efficacy of antioxidants in any given specimens. It was observed in a study by McAnlis et al. that 225 g fried onions rich in polyphenols increased plasma total antioxidant activity in humans,[33] while we observed that PSPL poly-

phenols didn't enhance plasma total antioxidant status. The direct radical quenching activity of polyphenols in vivo has been questioned because of their relatively low circulating concentrations as compared to plasma uric and ascorbic acid.

In addition to being annihilated by antioxidant defense, escaped reactive oxidant species can attack macromolecules and thereby cause pathogenesis of some diseases. For example, radical-mediated DNA damages are associated with carcinogenesis and oxidized LDL involve in atherogenesis. A growing body of evidence from in vitro, preclinical, and clinical studies suggested that polyphenols including flavonoids could protect LDL, DNA, protein, and lipid against oxidation.[18, 21, 34, 35] In this study, neither LPD nor 200 g/d PSPL altered the magnitudes of in vivo lipid peroxidation in apparently healthy individuals. In this study, the TBARS assay employed to assess MDA+4-HNE might be inadequate to reveal magnitude of in vivo lipid peroxidation because of appreciated interferences from bilirubin, sugar, and other factors. Interestingly, PSPL antioxidants decreased urinary excretion of DNA oxidation products temporarily in subjects in the PSPL group after the first week, but not after the second week. Similarly, polyphenols in onions and green tea diminished urinary 8-OHdG excretion in humans.[39, 41] Although these results of decreased urinary 8-OHdG excretion could be interpreted as a decrease in oxidant-induced DNA damage via antioxidative actions of polyphenols, it might simply suggest deceased capacities of DNA repairing mechanisms. In contrast to evidence from in vitro studies indicating antioxidant activity of polyphenols, our results suggested that antioxidant actions of PSPL polyphenols or other constituents might not be effective to diminish magnitudes of DNA and lipid oxidation in young healthy individuals when their endogenous antioxidant defense system is adequate to minimize *in vivo* oxidant-induced damages.[35-37] In vitro studies revealed, that polyphenols act as an oxygen radical scavenger as they enhance the resistance of LDL against Cu^{2+}-induced oxidation [38-43]. Our study showed significantly increased LDL lag time after PSPL consumption for 2 weeks. Further, antioxidant actions of PSPL polyphenols that might be too subtle to be detected in a Cu^{2+}-induced ex vivo oxidation model could be unmasked with *in vitro* addition of antioxidants.[21] The interactive effects among PSPL constituents, such as polyphenols and carotenoids, on antioxidant defense and oxidative stress remain to be investigated.

In conclusion, 902 mg of polyphenols in 200 g/d PSPL could be bioavailable and enhance glutathione status and decrease LDL oxidation. However, their antioxidant actions might not be sufficiently potent to modulate overall antioxidant defense in young healthy individuals.

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AUTHOR DISCLOSURES

Chiao-Ming Chen, Ya-Ling Lin, C-Y Oliver Chen, Ching-Yun Hsu, Ming-Jer Shieh and Jen-Fang Liu, no conflicts of interest.

REFERENCES

1. Arts IC, Hollman PC, Feskens EJ, Bueno de Mesquita HB, Kromhout D. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. Am I Clin Nutr. 2001;74(2):227-32.
2. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet. 1993;23;342(8878):1007-11.
3. Huxley RR, Neil HA. The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. Eur J Clin Nutr. 2003;57(8):904-8.
4. Jiang R, Manson JE, Stampfer MJ, Liu S, Willett WC, Hu FB. Nut and peanut butter consumption and risk of type 2 diabetes in women. JAMA. 2002;288(20):2554-60.
5. Tobias M, Turley M, Stefanogiannis N, Vander Hoorn S, Lawes C, Mhurchu CN, et al. Vegetable and fruit intake and mortality from chronic disease in New Zealand. Australian and New Zealand Journal of Public Health. 2006;30(1):26-31.
6. USA C, WHO. Promoting fruit and vegetable consumption around the world. http://wwwwhoint/dietphysicalactivity/fruit/en/indexhtml 2007;Visit on June 18.
7. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 1996;20(7):933-56.
8. Middleton E, Jr. Effect of plant flavonoids on immune and inflammatory cell function. Adv Exp Med Biol. 1998;439:175-82.
9. Duthie SJ, Johnson W, Dobson VL. The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimdines) and growth in human cells. Mutat Res. 1997;390(1-2):141-51.
10. Chu YF, Sun J, Wu X, Liu RH. Antioxidant and antiproliferative activities of common vegetables. J Agric Food Chem. 2002;50(23):6910-6.
11. Mosha TC, Gaga HE. Nutritive value and effect of blanching on the trypsin and chymotrypsin inhibitor activities of selected leafy vegetables. Plant Foods Hum Nutr. 1999;54(3):271-83.
12. Yan-Hwa Chu C-LCaH-FH. flavonoid content of several vegetables and their antioxidant activity. J Sci Food Agric. 2000;80:561-6.
13. Islam MS, Yoshimoto M, Yahara S, Okuno S, Ishiguro K, Yamakawa O. Identification and characterization of foliar polyphenolic composition in sweetpotato (Ipomoea batatas L.) genotypes. J Agric Food Chem. 2002;50(13):3718-22.
14. Philpott M, Gould KS, Lim C, Ferguson LR. In situ and in vitro antioxidant activity of sweetpotato anthocyanins. J Agric Food Chem. 2004;52(6):1511-3.
15. Yoshimoto M, Yahara S, Okuno S, Islam MS, Ishiguro K, Yamakawa O. Antimutagenicity of mono-, di-, and tricaffeoylquinic acid derivatives isolated from sweetpotato (Ipomoea batatas L.) leaf. Biosci Biotechnol Biochem. 2002;66(11):2336-41.
16. Chen CM, Li SC, Lin YL, Hsu CY, Shieh MJ, Liu JF. Consumption of purple sweet potato leaves modulates human immune response: T-lymphocyte functions, lytic activity of natural killer cell and antibody production. World J Gastroenterol. 2005;11(37):5777-81.

17. Chang WH, Chen CM, Hu SP, Kan NW, Chiu CC, Liu JF. Effect of purple sweet potato leaf consumption on the modulation of the antioxidative status in basketball players during training. Asia Pac J Clin Nutr. 2007;16(3):455-61.
18. Chen CY, Milbury PE, Collins FW, Blumberg JB. Avenanthramides are bioavailable and have antioxidant activity in humans after acute consumption of an enriched mixture from oats. J Nutr. 2007;137(6):1375-82.
19. Singleton VL RJ. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16:144-58.
20. Milne DB, Botnen J. Retinol, alpha-tocopherol, lycopene, and alpha- and beta-carotene simultaneously determined in plasma by isocratic liquid chromatography. Clin Chem. 1986;32(5):874-6.
21. Chen CY, Milbury PE, Lapsley K, Blumberg JB. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. J Nutr. 2005;135(6):1366-73.
22. Department of Health EY, R.O.C., editor. Nutrients database in Taiwan. Taiwan; 1998.
23. Fang YZ, Yang S, Wu G, editors. Free radicals, antioxidants, and nutrition. Nutrition (Burbank, Los Angeles County, Calif; 2002 Oct.
24. Tang S-C. Studies on the Antioxidative Capacity of Taiwan Indigenous Red Vegetables Extracts. Master Thesis Taipei Medical University. 2000.
25. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 2004;79(5):727-47.
26. Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr. 2005;81(1 Suppl):230S-42S.
27. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr. 2000;130(8S Suppl):2073S-85S.
28. Prentice RL, Willett WC, Greenwald P, Alberts D, Bernstein L, Boyd NF, et al. Nutrition and physical activity and chronic disease prevention: research strategies and recommendations. J Natl Cancer Inst. 2004;96(17):1276-87.
29. Luceri C, Caderni G, Sanna A, Dolara P. Red wine and black tea polyphenols modulate the expression of cycloxygenase-2, inducible nitric oxide synthase and glutathione-related enzymes in azoxymethane-induced f344 rat colon tumors. J Nutr. 2002;132(6):1376-9.
30. Ameho CK, Chen CY, Smith D, Sánchez-Moreno C, Milbury PE, Blumberg JB. Antioxidant activity and metabolite profile of quercetin in vitamin E depleted rats. J Nutr Biochem. 2008 ;19(7):467-74
31. Rimbach G, Gohil K, Matsugo S, Moini H, Saliou C, Virgili F, et al. Induction of glutathione synthesis in human keratinocytes by Ginkgo biloba extract (EGb761). Biofactors. 2001;15(1):39-52.
32. Scharf G, Prustomersky S, Knasmuller S, Schulte-Hermann R, Huber WW. Enhancement of glutathione and g-glutamylcysteine synthetase, the rate limiting enzyme of glutathione synthesis, by chemoprotective plant-derived food and beverage components in the human hepatoma cell line HepG2. Nutr Cancer. 2003;45(1):74-83.
33. McAnlis GT, McEneny J, Pearce J, Young IS. Absorption and antioxidant effects of quercetin from onions, in man. Eur J Clin Nutr. 1999;53(2):92-6.
34. Boyle SP, Dobson VL, Duthie SJ, Kyle JA, Collins AR. Absorption and DNA protective effects of flavonoid glycosides from an onion meal. Eur J Nutr. 2000;39(5):213-23.
35. Kim HY, Kim OH, Sung MK. Effects of phenol-depleted and phenol-rich diets on blood markers of oxidative stress, and urinary excretion of quercetin and kaempferol in healthy volunteers. J Am Coll Nutr. 2003;22(3):217-23.
36. Moller P, Vogel U, Pedersen A, Dragsted LO, Sandstrom B, Loft S. No effect of 600 grams fruit and vegetables per day on oxidative DNA damage and repair in healthy nonsmokers. Cancer Epidemiol Biomarkers Prev. 2003;12 (10):1016-22.
37. Jacob RA, Aiello GM, Stephensen CB, Blumberg JB, Milbury PE, Wallock LM, et al. Moderate antioxidant supplementation has no effect on biomarkers of oxidant damage in healthy men with low fruit and vegetable intakes. J Nutr. 2003;133(3):740-3.
38. Pietta PG. Flavonoids as antioxidants. J Nat Prod. 2000; 63(7):1035-42.
39. Miura Y, Chiba T, Miura S, Tomita I, Umegaki K, Ikeda M, et al. Green tea polyphenols (flavan 3-ols) prevent oxidative modification of low density lipoproteins: an ex vivo study in humans. J Nutr Biochem. 2000;11(4):216-22.
40. Hirano R, Osakabe N, Iwamoto A, Matsumoto A, Natsume M, Takizawa T, et al. Antioxidant effects of polyphenols in chocolate on low-density lipoprotein both in vitro and ex vivo. J Nutr Sci Vitaminol (Tokyo). 2000;46(4):199-204.
41. Chen YY, Liu JF, Chen CM, Chao PY, Chang TJ. A study of the antioxidative and antimutagenic effects of Houttuynia cordata Thunb. using an oxidized frying oil-fed model. J Nutr Sci Vitaminol (Tokyo). 2003;49(5):327-33.
42. Mursu J, Voutilainen S, Nurmi T, Helleranta M, Rissanen TH, Nurmi A, et al. Polyphenol-rich phloem enhances the resistance of total serum lipids to oxidation in men. J Agric Food Chem. 2005;53(8):3017-22.
43. Chang S, Tan C, Frankel EN, Barrett DM. Low-density lipoprotein antioxidant activity of phenolic compounds and polyphenol oxidase activity in selected clingstone peach cultivars. J Agric Food Chem. 2000;48(2):147-51.