行政院國家科學委員會專題研究計畫 成果報告

甘蔗葉生物活性成分鑑定與保護血管內皮細胞氧化傷害作用
研究成果報告(精簡版)

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計畫參與人員：此計畫無其他參與人員

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行政院國家科學委員會補助專題研究計畫 ■ 期末成果報告

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成果報告類型(依經費核定清單規定繳交)：☑精簡報告　□完整報告

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執行單位：嘉南藥理科技大學 食品科技系

中　華　民　國　98　年　7　月 30 日

摘要

本研究為探討甘蔗葉生物活性成分鑑定與保護血管內皮細胞氧化傷害作用，原計畫分成三年完成。第一年計畫為探討甘蔗葉萃取物於體外試驗之抗突變效應及其對細胞性發炎反應之調控性。 結果顯示，甘蔗葉熱水萃取物在0-10mg/plate沒有顯著的致突變作用也可以有效抑制間接突變劑benzo[a]pyrene（BP）;和直接突變劑N-methyl-N'- nitrosoguanidine（MNNG），對鼠傷寒沙門氏菌TA98和TA100的致突變作用。此外，甘蔗葉熱水萃取物在0-0.4 mg/ml 可以抑制超氧陰離子，捕捉一氧化氮自由基，以及降低脂質氧化傷害。在0-1.0 mg/ml，甘蔗葉熱水萃取物可以抑制 脂多醣（LPS）刺激巨噬細胞發炎作用，且NO生成濃度隨甘蔗葉熱水萃取物濃度的增加而減少。利用高效能液相層析分析，甘蔗葉熱水萃取物含有許多多酚成分，如caffeic acid, ferulic acid, apigenin, vitexin。這些數據顯示，應用甘蔗葉熱水萃取物可能有助於體內抗突變和抗氧化作用。

關鍵字：甘蔗葉，抗突變，抗氧化

Abstract

In this three-year project, the major purpose is to investigate of the bioactivities of sugarcane leaves in vivo and in vitro. The major goal of the first year is to study the protective effects of sugarcane leaves on mutation of *Salmonella* typhimurium TA98 and TA100 and cellular the inflammatory reaction. However, in this study, the effects of a water extract of sugarcane leaves (WSL) on antimutation and nitric oxide production were investigated. The results showed that WSL inhibited the mutagenicity of benzo[a]pyrene (BP), an indirect mutagen; and N-methyl-N'-nitrosoguanidine (MNNG), a direct mutagen toward *Salmonella* typhimurium TA98 and TA100. In addition, WSL, in the range of 0–0.4 mg/ml, showed superoxide inhibiting, NO scavenging, and reducing activity, as well as decreased lipid oxidative damage. In the range of 0–1.0 mg/ml, the inhibitory effect of WSL on NO generation in lipopolysaccharide (LPS) stimulated macrophages increased with increasing concentrations. A high performance liquid chromatography analysis revealed that the polyphenolic constituents such as caffeic acid, ferulic acid, apigenin, and vitexin were present in the WSL. These data suggested that the WSL exhibiting biological activities could contribute to antimutation as well as antioxidation.

Keywords: sugarcane leaves, anti-mutation, anti-oxidant

Introduction

Lipid oxidations not only play a destructive role, decreasing the quality of the food, they can also contaminate the food with their harmful by-products. At the same time, lipid oxidation induced by reactive oxygen radicals (e.g. reactive oxygen species and reactive nitrogen species) will lead to cellular damage and promote the pathological progression of carcinogenesis, atherosclerosis, and diabetes. Therefore, to decrease the oxidation of lipids, various antioxidants have been used to protect the lipids in foods from oxidation. In addition to lipid oxidation, numerous reports revealed that various environmental mutagens present in contaminative foods exhibit different mechanisms to oxidative stress and induce DNA mutation production. For example, benzo[a]pyrene (BP) is a known organic tetracyclic hydrocarbon for carcinogenicity in the lung. In addition, N-methyl-N'-nitro-N nitrosoguanidine (MNNG), another strong environmental mutagen, is generally thought to induce direct methylation of DNA by methyldiazohydroxide. These damages, if not prevented, may result in DNA mutation and eventually increase the risk of tumors progression. Except to form direct adducts with DNA, these toxic mutagens may be metabolized and further induce harmful free radicals generation in cells, which also destroy the biological molecules (e.g. lipids and proteins) and promote the production of cell damages.

Numerous investigations on the protective roles of sugarcane (*Sacharum officinarum* L.) derivatives showed that the extracts of sugarcane exhibit antiproliferative, antioxidative, and hepatoprotective abilities. These studies suggested that the polyphenols and flavonoids present in sugarcane could contribute to these health benefits. In fact, previous studies have revealed that, in addition to act as biological oxidation inhibitors, natural antioxidants could also reduce mutation by preventing mutagen activation. However, in spite of many studies on sugarcane, there have been few studies focusing on the bioactive activity of the sugarcane leaves against mutation and oxidation production. To use various phytochemical components for decrease the mutagen activation and oxidative damage is a popular and important step against cancers and degenerative diseases. Consequently, a better understanding of the bifunctional properties of naturally occurring materials has become a critical goal. Thus, the aim of this study was to investigate the defense capacity of sugarcane leaves on mutagen induced mutation, and the protective effects provided against oxidative stress damage *in vitro*.

Results

Previous studies have indicated that the free radical scavenging activities observed in sugarcane may be due to various natural polyphenolics (e.g. caffeic acid, ferulic acid, apigenin, vitexin) which displayed strong antioxidation effects; thus caffeic acid, ferulic acid, apigenin, and vitexin were selected as fingerprint markers for HPLC analysis of WSL, and

the chromatograph was shown as in Figure 1. In screening the genotoxicity for plant extracts, the Ames assay is a commonly method to determine the potential mutagenicity. In the present study, if a lethal toxicity occurred in a test treated with WSL, the results of mutagenicity assay would be affected and confuse the numbers of revertants of TA 98 and TA 100. Therefore, the cytotoxicity of WSL against *S. typhimurium* TA 98 and TA 100, respectively, was determined first. The WSL tested in our present study in the range of 1–10 mg/plate did not show any toxicity against TA 98 and TA 100, respectively, indicating that WSL did not exhibit toxicity toward TA 98 and TA 100 (data not shown). Further, the mutagenicity of WSL was measured by comparing the ratio of induced revertants and spontaneous revertants in plates. According to Table 1, in the range of 1–10 mg/plate, WSL did not exhibit significant effect on colony numbers toward *S. typhimurium* TA 98 and toward *S. typhimurium* TA 100 with or without S9 activation. However, no significant mutagenicity of WSL was found for TA98 and TA100 ($p < 0.05$) at concentrations from 1–10 mg/plate as compared to control group. Hence, the dose of 1–10 mg/plate was selected for the antimutagenic assay. For testing the antimugenicity of WSL, BP and MNNG were used as indirect and direct acting mutagens, respectively, to induce *S. typhimurium* TA 98 and TA 100 mutation.

Table 2 shows the antimutagenicity of WSL against BP and MNNG induced mutation toward *S. typhimurium* TA 98 and TA 100 with or without S9 activation. In the range of 1–10 mg/plate, WSL showed dose-dependent inhibitory effects against the mutagenicity of BP and MNNG toward *S. typhimurium* TA98 with or without S9 activation. WSL at 1–10 mg/plate showed 1–42% and 2–31% inhibitory effect against BP and MNNG induced TA 98 mutation, respectively. Meanwhile, WSL showed 1–51% and 0-27% inhibitory effect against BP and MNNG induced TA 100 mutation. Apparently, WSL showed a higher inhibitory effect against indirect mutation toward TA 98 and TA 100 compared with against direct mutation.

Table 3 shows superoxide scavenging, NO scavenging, and total phenolics of the WSL. In the range of 0.05 - 0.4 mg/ml, WSL showed 42.5 - 89.8% scavenging activity on superoxide. For the other columns of Table 3, WSL also exhibited a concentration-dependent increase in the effect of NO scavenging, with 17.7 - 53.7% inhibitory activity. However, as shown in Table 3, the contents of total phenolics in the WSL were expressed in gallic acid equivalents. The results showed that the WSL at 0.4 mg dry extract/ml contained amounts of total phenolics equal to 40.2 μg gallic acid/ml. In other words, the levels of total phenolics in the WSL were 100.5 mg/g extract. Further, the correlation between the superoxide scavenging and the total

phenolics contents was determined by linear regression analysis. While a positive correlation (r^2= 0.895) between the superoxide scavenging and the total phenolics contents of WSL was found, the NO scavenging correlated even better (r^2= 0.934) with the total phenolics content of the WSL. This implied that the radical scavenging effects of the WSL might be attributed to the total phenolic constituents of WSL.

Table 4 shows the liposome protection, reducing power, and the total flavonoids of the WSL. Lipid peroxidation is a harmful process, producing toxic aldehyde and promoting cellular pathological metabolism. In this study, liposome protection was used as an index to assay the protective action of the WSL on lipid oxidation. WSL in the range of 0.05-0.4 mg/ml exhibited a dose-dependent protective effect, 4.4 - 42.3 %, on the liposome damage induced by the Fe^{3+}/H_2O_2 reaction. Meanwhile, reducing activity of natural products, which can be regarded as a reduced hydrogen donating capacity, can usually achieved by terminating the radicals' chain reaction and thus minimizing their damage. The results on WSL on this effect were compared to that of ascorbic acid. As shown in Table 4, the reducing powder of WSL was a concentration-dependent and at 0.4 mg/ml was as high as 34.5 μg/ml of ascorbic acid. Further, the contents of total flavonoids in the WSL were expressed in rutin equivalents. The results showed that the WSL at 0.4 mg dry extract/ml contained amounts of total phenolics equal to 14.1 μg rutin/ml. In other words, the levels of total flavonoids in the WSL were 32.3 mg/g extract. Overall speaking, there was a positive linear correlation (r^2= 0.994) between liposome protection and the total flavonoids of WSL. Meanwhile the reducing powder also correlated well (r^2= 0.987) with the total flavonoids of WSL.

In cellular model, the NO inhibitory activity of the WSL was determined by using the LPS activated macrophages to produce mass NO radicals, then measured as nitrite in the culture medium by Griess reaction. As shown in Figure 2, the WSL, in the range of 0.1-0.8 mg/ml, reduced the NO production of activated macrophages in a dose-dependent manner. WSL at the concentration of 0.4 and 0.8 mg/ml, the levels of NO in medium were decreased to 14.1 and 2.5%, respectively, of that observed in LPS alone. This suggests that WSL could be a potential NO scavenger. In addition, no cell toxicity was observed with WSL, as measured by the MTT cell viability test. The results obviously indicated that WSL could reduce RNS generation in stimulated macrophages.

Discussion

In the present study, neither toxicity and nor mutagenicity was found toward *S. typhimurium* TA 98 and TA 100 in the presence of sugarcane leaves with or without S9 mix. However, no previous report found sugarcane leaves might contain mutagenic phytochemicals, and also the mutagenic activity of sugarcane leaves was not observed in this study. Regarding the mechanisms of

antimutagenesis, there are different pathways including: (1) inactivation of mutagens, (2) inhibition of metabolic activation of promutagens, and (3) inactivation of activated mutagens. In this study, it is well known that BP and MNNG are indirect and direct mutagens, respectively. According to the data presented in Table 2, WSL showed the inhibitory effects on the mutagenicity of BP toward TA98 and TA100 with liver S9 mix. Meanwhile, WSL also clearly decrease the mutagenicity of MNNG, which is a direct-acting mutagen. These data implied that WSL might directly inactivate BP and MNNG mutagens to decrease the number of revertants in *S. typhimurium*. In other word, the conjugate formation between WSL and the toxic electrophiles might be an important detoxification mechanism in the present study. Apart from these, the antimutagenic activities of WSL might also be attributed to inhibit metabolic activation and decrease genotoxic reactive intermediates production, which would further indirectly decrease cellular oxidative stress and prevent the production of mutation. And the correlation between the polyphenolic contents of vegetables and their free radical inhibition activity is well documented. As shown in Table 3, the WSL exhibited antioxidant activity by scavenging superoxide radical and nitric oxide. When comparing the radical scavenging activity, it is found that the total phenolics of WSL has more correlation with its nitric oxide scavenging (r^2= 0.934) than its superoxide scavenging (r^2= 0.895). This indicated that the nitric oxide scavenging activity of WSL was more active than its superoxide inhibition. Therefore, the biological radical scavenging effects of the WSL could be attributed to these bioactive phenolic constituents present in WSL. In summary, as mentioned above, WSL showed antimutation, antioxidation, and decrease reactive nitrogen species stress. These activities could be closely contributed to its total flavonoid and polyphenolic constituents. Other unknown active components in the WSL could also play various critical roles in its protective effects. Although these results revealed the possible protective effects of the WSL against mutagen induced mutation and inflammatory diseases, further investigations of the nutritional and physiological effects of sugarcane leaves are still intensively required.

References

Ames, B. N. (1983). Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science*, *221*(4617), 1256-1264.

de Oliveira, A. C., Valentim, L. B., Silva, C. A., Bechara, E. J. H., de Barros, M. P., Mano, C. M., & Goularta, M. O. F. (2009). Total phenolic content and free radical scavenging activities of methanolic extract powders of tropical fruit residues. *Food Chemistry*, *115*(2), 469-475.

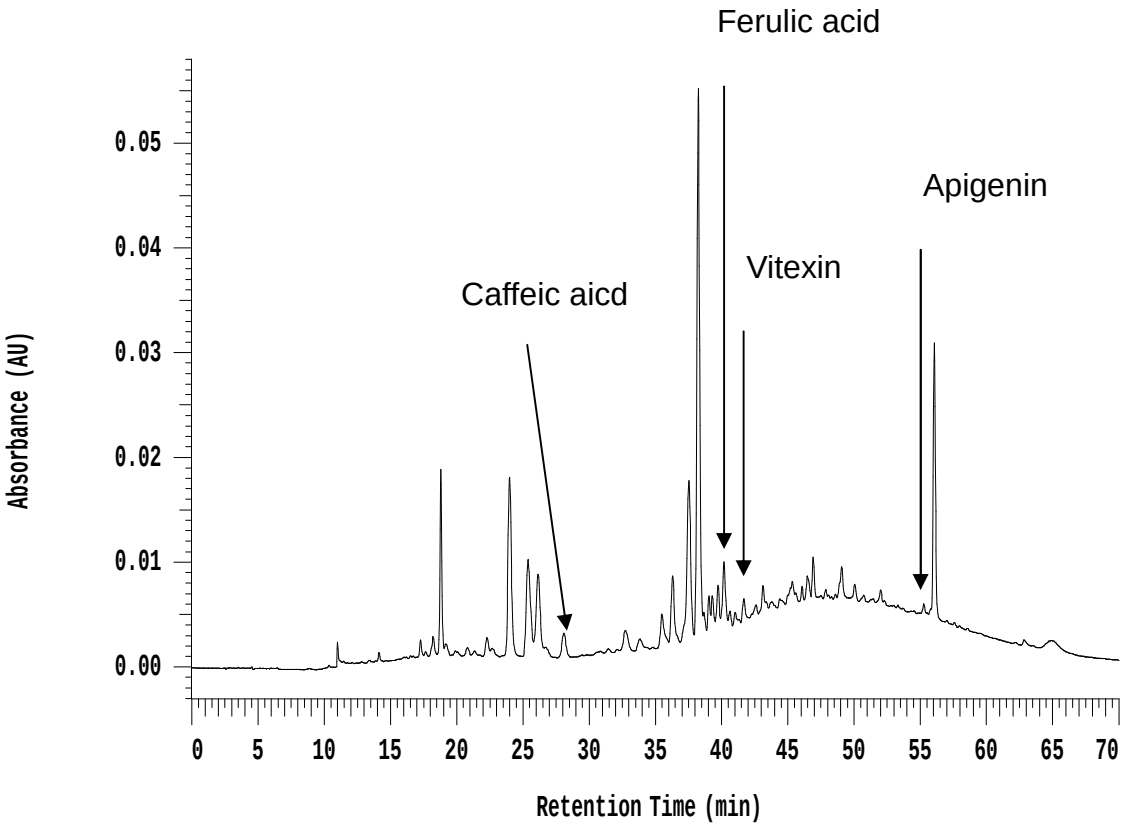

Figure 1. HPLC chromatogram of WSL

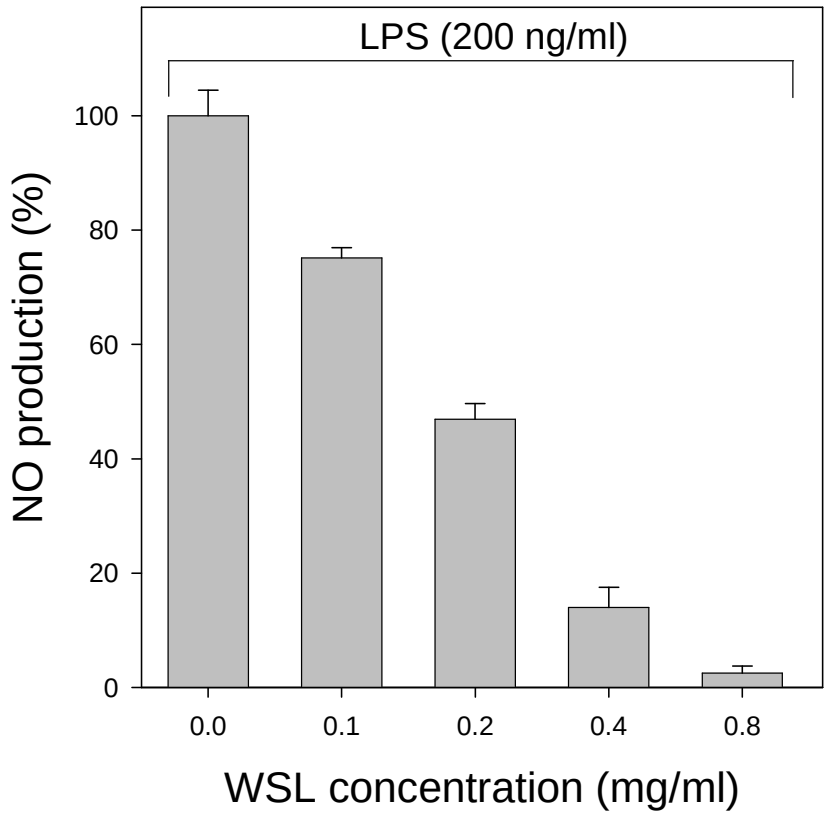

Figure 2. Effects of WSL on nitric oxide production in lipopolysaccharide stimulated macrophages. The data were displayed with mean ± S.D. of three experiments individually and analyzed by ANOVA ($p < 0.05$).

Table 1. The mutagenicity of WSL toward *S. typhimurium* TA98 and TA100 with and without S9 mix [A]

Sanple (mg/plate)	His+ revertants/plate (% of spontaneous) [B]					
	TA98 -S9			TA98 +S9		
Spontaneous group		29 ± 6 (100) [a]			35 ± 4 (100) [a]	
1.0		26 ± 3 (90) [a]			30 ± 2 (86) [a]	
2.5		41 ± 7 (141) [a]			41 ± 4 (117) [a]	
5.0		40 ± 10 (138) [a]			26 ± 7 (74) [a]	
10.0		39 ± 11 (134) [a]			45 ± 9 (129) [a]	
	TA100 -S9			TA100 +S9		
Spontaneous group		128 ± 14 (100) [a]			135 ± 13 (100) [a]	
1.0		119 ± 20 (93) [a]			131 ± 7 (97) [a]	
2.5		142 ± 11 (111) [a]			122 ± 10 (90) [a]	
5.0		126 ± 15 (98) [a]			143 ± 12 (106) [a]	
10.0		142 ± 8 (111) [a]			145 ± 9 (107) [a]	

[A] Data are means ± SD of three plates. Values with different superscripts in a column are significantly different ($p < 0.05$). [B] % of spontaneous=[(no. of his^+revertants in the presence of sample)/(no. of spontaneous revertants)]×100. The number of spontaneous revertants was determined without samples and mutagen

Table 2. The antimutagenicity of WSL toward *S. typhimurium* TA98 and TA100 in the presence of mutagens with and without S9 mix [A]

Sanple (mg/plate)	His+ revertants/plate (% of inhibition) [B]					
	TA98 +BP +S9			TA98 +MNNG –S9		
0		1723 ± 64 (0) [a]			726 ± 24 (0) [a]	
1.0		1701 ± 49 (1) [a]			711 ± 19 (2) [a]	
2.5		1682 ± 71 (2) [a]			688 ± 33 (5) [ab]	
5.0		1629 ± 55 (6) [a]			669 ± 25 (8) [ab]	
10.0		1008 ± 58 (42) [c]			508 ± 41 (31) [c]	
	TA100 +BP+S9			TA100 +MNNG -S9		
0		1802 ± 53 (0) [a]			984 ± 31 (0) [a]	
1.0		1781 ± 63 (1) [a]			980 ± 12 (0) [a]	
2.5		1729 ± 48 (4) [a]			929 ± 23 (6) [a]	
5.0		1482 ± 61 (19) [b]			842 ± 22 (17) [b]	
10.0		957 ± 58 (51) [d]			757 ± 8 (27) [c]	

[A] Data are means ± SD of three plates. Values with different superscripts in a column are significantly different ($p < 0.05$). BP, benzo[a]pyrene. MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. [B] % of inhibition = {1-[(no. of revertants with mutagen and sample - no. of spontaneous revertants)/(no. of revertants with mutagen -no. of spontaneous revertants)]}×100. The number of spontaneous revertants was determined without samples and mutagen.

Table 3. Total phenolics and free radical scavenging capacity of WSL

WSL (mg/ml)	Superoxide scavenging (%)	NO scavenging (%)	Total phenolics (μg/ml)
0.05	42.5 ± 7.5 [a]	17.7 ± 2.3 [a]	5.0 ± 0.3 [a]
0.1	53.1 ± 6.1 [a]	30.5 ± 2.2 [b]	11.8 ± 0.9 [b]
0.2	76.9 ± 4.2 [b]	40.6 ± 1.4 [c]	19.8 ± 1.7 [c]
0.4	89.8 ± 12.1 [b]	53.7 ± 1.8 [d]	40.2 ± 2.4 [d]

Results are mean ± SD for n = 3. Values with different superscripts in a column are significantly different ($p < 0.05$).

Table 4. The liposome protection, reducing powder, and total flavonoids of WSL

WSL (mg/ml)	Liposome protection (%)	Reducing powder (μg/ml)	Total flavonoids (μg/ml)
0.05	4.4 ± 0.9 [a]	5.7 ± 0.4 [a]	2.0 ± 0.2 [a]
0.1	10.1 ± 2.3 [a]	10.2 ± 4.0 [a]	3.3 ± 0.3 [b]
0.2	25.4 ± 5.1 [b]	17.3 ± 1.5 [b]	7.9 ± 0.7 [c]
0.4	42.3 ± 2.2 [c]	34.5 ± 2.5 [c]	14.1 ± 2.0 [d]

Results are mean ± SD for n = 3. Values with different superscripts in a column are significantly different ($p < 0.05$).